09-18-00

A

Customer No. 000959

Case Docket No. UMV-1226CPPCUS

THE ASSISTANT COMMISSIONER FOR PATENTS
Box Patent Application
Washington, D.C. 20231

"Express Mail" Mailing Label Number EL 373206919 US
Date of Deposit September 15, 2000

I hereby certify that this transmittal letter and the papers referred to as being enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

Signature
Ilidio P. Cardoso
Please Print Name of Person Signing

Sir:

Transmitted herewith for filing is the patent application of

Inventor(s): Patrick J. Venta, George J. Brewer, Vilma Yuzbasiyan-Gurkan and William D. Schall

For: DNA ENCODING CANINE VON WILLEBRAND FACTOR AND METHODS OF USE

Enclosed are:

☒ 20 pages of specification, 5 pages of claims, 1 pages of abstract.

☒ 16 pages of drawings.

☒ Unexecuted Declaration, Petition and Power of Attorney (6 pages).

☒ Executed (copy) of a verified statement to establish small entity status under 37 C.F.R. 1.9 and 37 C.F.R. 1.27

☒ A Preliminary Amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning the number next following the highest numbered original claims in the prior application.)

The filing fee has been calculated as shown below:

	(Col. 1)	(Col. 2)	SMALL ENTITY			OTHER THAN SMALL ENTITY	
FOR:	NO. FILED	NO. EXTRA	RATE	FEE	OR	RATE	FEE
BASIC FEE	////////////////////////////////		//////////	$	OR	//////////	$ 690.00
TOTAL CLAIMS	29- 20	= 9	x 9=	$81.00	OR	x 18=	
INDEP. CLAIMS	12- 3	= 9	x 39	$351.00	OR	x 78	
☐ MULTIPLE DEPENDENT CLAIMS PRESENTED			+130	$	OR	+260	$
			TOTAL		OR	TOTAL	$1,122.00

* If the difference in Col. 2 is less than zero, enter "0" in Col. 2.

☐ Please charge my Deposit Account No. 12-0080 in the amount of $.
A duplicate copy of this sheet is enclosed.

☒ A check in the amount of $1,122.00 to cover the filing fee is enclosed.

☐ The Commissioner is hereby authorized to charge payment of the following fees associated with this communication or credit any overpayment to Deposit Account No. 12-0080.
A duplicate copy of this sheet is enclosed.

☐ Any additional filing fees required under 37 C.F.R. 1.16.

☐ Any patent application processing fees under 37 C.F.R. 1.17.

☐ The Commissioner is hereby authorized to charge payment of the following fees during the pendency of this application or credit any overpayment to Deposit Account No. 12-0080.
A duplicate copy of this sheet is enclosed.

- ☐ Any patent application processing fees under 37 C.F.R. 1.17.
- ☐ The issue fee set in 37 C.F.R. 1.18 at or before mailing of the Notice of Allowance, pursuant to 37 C.F.R 1.311(b).
- ☐ Any filing fees under 37 C.F.R. 1.16 for presentation of extra claims.

☐ A check in the amount of $_____ to cover the recording of assignment documents is also enclosed.

☒ The Commissioner is hereby authorized to charge any additional payment or credit any overpayment to Deposit Account No. 12-0080. A duplicate copy of this sheet is enclosed.

☒ Address all future communications (May only be completed by applicant, or attorney or agent of record) to DeAnn F. Smith, Esq. at **Customer Number: 000959** whose address is:

Lahive & Cockfield, LLP
28 State Street
Boston, Massachusetts 02109

Date: September 15, 2000

LAHIVE & COCKFIELD, LLP
Attorneys at Law

By _____
DeAnn F. Smith
Attorney for Applicant
28 State Street
Boston, MA 02109
(617) 227-7400
Telecopier (617) 742-4214

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s) or Patentee:	Patrick J. Venta et al.
Serial or Patent No.:	09/132,652
Filed or Issued:	August 11, 1998
For:	DNA Encoding Canine Von Willebrand Factor And Methods Of Use
Attorney Docket No.:	2115S01226CPB

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

[ ] the owner of the small business concern identified below:
[XX] an official of the small business concern empowered to act on behalf of the concern identified below:

Name Of Concern: VetGen, L.L.C.

Address Of Concern: 3728 Plaza Drive
Suite 1
Ann Arbor, Michigan 48108

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.1301-.1305, and referenced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that exclusive rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention described in

[ ] the specification filed herewith.
[XX] the application whose serial number is set forth above.
[ ] the patent set forth above.

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Signed: _____ Date: 11-12-98

Name Of Person Signing: John Duffendeck

Title Of Person If Other Than Owner: President, Chief Executive Officer

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: Patrick J. Venta, et al.

Serial No.: N/A

Filed: Herewith

For: *DNA ENCODING CANINE VON WILLEBRAND FACTOR AND METHODS OF USE*

Attorney Docket No.: UMV-1226CPPCUS

Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

"Express Mail" Mailing Label Number EL 373206919 US
Date of Deposit September 15, 2000

I hereby certify that this transmittal letter and the papers referred to as being enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

Signature
Ilidio P. Cardoso
Please Print Name of Person Signing

PRELIMINARY AMENDMENT

Dear Sir:

Prior to examination of the above-identified application, please amend the application as follows:

In the Specification

Please insert before the Background of the Invention, the following:

Related Applications

The present application claims priority to PCT patent application serial number PCT/US99/18153, filed on August 10, 1999, which claims priority to U.S. Patent No. 6,074,832, issued June 13, 2000.

In the Claims

Please delete claims 1-29 and add new claims 30-45 as follows.

30. An isolated nucleic acid molecule comprising a nucleotide sequence encoding mutated canine von Willebrand Factor polypeptide which causes canine von Willebrand's disease, wherein the nucleotide sequence is capable of hybridizing under high stringency conditions to the complementary sequence of the sequence of SEQ ID NO. 1 having a mutation at nucleotide 937.

31. A vector comprising the nucleic acid molecule of Claim 30.

32. A cell comprising the vector of Claim 31.

33. The isolated nucleic acid molecule of Claim 30, wherein the mutation at nucleotide 937 is a base deletion.

34. A method of detecting a canine von Willebrand Factor gene in a sample comprising the steps of:

a) contacting the sample with an oligonucleotide comprising contiguous nucleotides of the nucleic acid sequence of SEQ ID NO. 1 or complement thereof, having a mutation at nucleotide 937, and capable of specifically hybridizing with the canine von Willebrand Factor gene, under conditions favorable for hybridization of the oligonucleotide to any complementary sequence of nucleic acid in the sample; and

b) detecting hybridization, thereby detecting a canine von Willebrand Factor gene.

35. The method of Claim 34, further comprising the step of:

c) quantifying hybridization of the oligonucleotide to the complementary sequence.

36. The method of Claim 34, wherein the mutation at nucleotide 937 is a base deletion.

37. An assay kit for screening for a canine von Willebrand Factor gene comprising:

a) an oligonucleotide comprising contiguous nucleic acids of the nucleotide sequence of SEQ ID NO. 1 having a mutation at nucleotide 937, and capable of hybridizing with the nucleotide sequence encoding canine von Willebrand Factor;

b) reagents for hybridization of the oligonucleotide to a complementary nucleic acid sequence; and

c) container means for a)-b).

38. The assay kit of Claim 37, wherein the mutation at nucleotide 937 is a base deletion.

39. An assay kit for screening for a canine von Willebrand Factor gene comprising:

a) a oligonucleotide comprising contiguous nucleic acids of the nucleotide sequence that is complementary to the sequence of SEQ ID NO. 1 having a mutation at nucleotide 937, and capable of specifically hybridizing to the complementary nucleotide sequence;

b) reagents for hybridization of the oligonucleotide to a complementary nucleic acid sequence; and

c) container means for a)-b).

40. The assay kit of Claim 39, wherein the mutation at nucleotide 937 is a base deletion.

41. A method for detecting a mutated canine von Willebrand Factor gene in a canine DNA sample comprising the steps of:

a) amplifying the DNA sample by polymerase chain reaction to produce polymerase chain reaction products, wherein the polymerase chain reaction uses primers that produce a restriction site in a mutant allele but not in a normal allele, wherein the mutation in the mutant allele is a base deletion at nucleotide 937 of the gene encoding canine von Willebrand Factor;

b) digesting the polymerase chain reaction products with a restriction enzyme specific to the restriction site of the restriction site primer to produce DNA fragments; and

c) detecting the DNA fragments, thereby detecting a mutated canine von Willebrand Factor gene.

42. The method of Claim 41, wherein the DNA fragments are detected by gel electrophoresis.

43. The method of Claim 41, wherein the primers comprise the sequence of SEQ ID NOS: 23 and 25.

44. The method of Claim 41, wherein the restriction enzyme is *Mwo I.*

45. An oligonucleotide probe capable of detecting a mutation associated with canine von Willebrand's disease, wherein the mutation is a base deletion at nucleotide 937 of the nucleotide sequence encoding canine von Willebrand Factor polypeptide, wherein the nucleotide sequence is capable of hybridizing under high stringency conditions to the complementary sequence of the sequence of SEQ ID NO. 1.

CONCLUSION

In view of the amendments and remarks set forth above, it is respectfully submitted that this application is in condition for allowance. If there are any remaining issues or the Examiner believes that a telephone conversation with Applicants' Attorney would be helpful in expediting prosecution of this application, the Examiner is invited to call the undersigned at (617) 227-7400.

Respectfully submitted,

LAHIVE & COCKFIELD, LLP

DeAnn F. Smith
Reg. No. 36,683
Attorney for Applicants

28 State Street
Boston, Massachusetts 02109
Telephone: (617) 227-7400
Facsimile: (617) 742-4214

Date: September 15, 2000

DNA ENCODING CANINE VON WILLEBRAND FACTOR AND METHODS OF USE

RELATED APPLICATIONS

The present invention is a continuation-in-part of U.S. Serial No. 08/896,449, filed July 18, 1997, which claims priority from U.S. Serial No. 60/020,998, filed July 19, 1996, both hereby expressly incorporated by reference.

FIELD OF THE INVENTION

This invention relates generally to canine von Willebrand factor (vWF), and more particularly, to the gene encoding vWF as well as a genetic defect that causes canine von Willebrand's disease.

BACKGROUND OF THE INVENTION

In both dogs and humans, von Willebrand's disease (vWD) is a bleeding disorder of variable severity that results from a quantitative or qualitative defect in von Willebrand factor (vWF) (Ginsburg, D. et al., *Blood* 79:2507-2519 (1992); Ruggeri, Z.M., et al., *FASEB J* 7:308-316 (1993); Dodds, W.J., *Mod Vet Pract* 681-686 (1984); Johnson, G.S. et al., *JAVMA* 176:1261-1263 (1988); Brooks, M., *Probl In Vet Med* 4:636-646 (1992)). This clotting factor has two known functions, stabilization of Factor VIII (hemophilic factor A) in the blood, and aiding the adhesion of platelets to the subendothelium, which allows them to provide hemostasis more effectively. If the factor is missing or defective, the patient, whether human or dog, may bleed severely.

The disease is the most common hereditary bleeding disorder in both species, and is genetically and clinically heterogenous. Three clinical types, called 1, 2, and 3 (formerly I, II, and III; see Sadler, J.E. et al., *Blood* 84:676-679 (1994) for nomenclature changes), have been described. Type 1 vWD is inherited in a dominant, incompletely penetrant fashion. Bleeding appears to be due to the reduced level of vWF rather than a qualitative difference. Although this is the most common form of vWD found in most mammals, and can cause serious bleeding problems, it is generally less severe than the other two types. In addition, a relatively inexpensive vasopressin analog (DDAVP) can help alleviate symptoms (Kraus, K.H. et al., *Vet Surg* 18:103-109 (1989)).

In Type 2 vWD, patients may have essentially normal levels of vWF, but the factor is abnormal as determined by specialized tests (Ruggeri, Z.M., et al., *FASEB J* 7:308-316 (1993); Brooks, M., *Probl In Vet Med* 4:636-646 (1992)). This type is

also inherited in a dominant fashion and has only rarely been described in dogs (Turrentine, M.A., et al., *Vet Clin North Am Small Anim Pract* 18:275 (1988)).

Type 3 vWD is the most severe form of the disease. It is inherited as an autosomal recessive trait, and affected individuals have no detectable vWF in their blood. Serious bleeding episodes require transfusions of blood or cryoprecipitate to supply the missing vWF. Heterozygous carriers have moderately reduced factor concentrations, but generally appear to have normal hemostasis.

Scottish terriers have Type 3 vWD (Dodds, W.J., *Mod Vet Pract* 681-686 (1984); Johnson, G.S. et al., *JAVMA* 176:1261-1263 (1988)). Homozygotes have no detectable vWF and have a severe bleeding disorder. Heterozygotes have reduced levels of the factor, and are clinically normal (Brooks, M. et al., *JAVMA* 200:1123-1127 (1992)). The prevalence of vWD among Scottish terriers including both heterozygotes and homozygotes has been variously estimated from 27-31% (Stokol, T. et al., *Res. Vet. Sci.* 59:152-155 (1995); Brooks, M., *Proc. 9th ACVIM Forum* 89-91 (1991)).

Currently, detection of affected and carrier Scottish terrier dogs is done by vWF antigen testing (Benson, R.E. et al., *Am J Vet Res* 44:399-403 (1983); Stokol, T. et al., *Res. Vet. Sci.* 59:152-155 (1995)) or by coagulation assays (Rosborough, T.K. et al., *J. Lab. Clin. Med.* 96:47-56 (1980); Read, M.S. et al., *J. Lab. Clin. Med.* 101:74-82 (1983)). These procedures yield variable results, as the protein-based tests can be influenced by such things as sample collection, sample handling, estrous, pregnancy, vaccination, age, and hypothyroidism (Strauss, H.S. et al., *New Eng J Med* 269:1251-1252 (1963); Bloom, A.L., *Mayo Clin Proc* 66:743-751 (1991); Stirling, Y. et al., *Thromb Haemostasis* 52:176-182 (1984); Mansell, P.D. et al., *Br. Vet. J.* 148:329-337 (1992); Avgeris, S. et al., *JAVMA* 196:921-924 (1990); Panciera, D.P. et al., *JAVMA* 205:1550-1553 (1994)). Thus, for example, a dog that tests within the normal range on one day, can test within the carrier range on another day. It is therefore difficult for breeders to use this information.

It would thus be desirable to provide the nucleic acid sequence encoding canine vWF. It would also be desirable to provide the genetic defect responsible for canine vWD. It would further be desirable to obtain the amino acid sequence of canine vWF. It would also be desirable to provide a method for detecting carriers of the defective vWF gene based on the nucleic acid sequence of the normal and defective vWF gene.

SUMMARY OF THE INVENTION

The present invention provides a novel purified and isolated nucleic acid sequence encoding canine vWF. Nucleic acid sequences containing the mutations that cause vWD in Scottish terriers, Doberman pinschers, Shetland sheepdogs, Manchester terriers and Poodles are also provided. The nucleic acid sequences of the present invention may be used in methods for detecting carriers of the mutation that causes vWD. Such methods may be used by breeders to reduce the frequency of the disease-causing allele and the incidence of disease. In addition, the nucleic acid sequence of the canine vWF provided herein may be used to determine the genetic defect that causes vWD in other breeds as well as other species.

Additional objects, advantages, and features of the present invention will become apparent from the following description, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and by referencing the following drawings in which:

Figures 1A-1C is the nucleic acid sequence of the canine von Willebrand factor of the present invention (SEQ ID NO: 1);

Figures 2A-2C is a comparison of the human and canine prepro-von Willebrand factor amino acid sequences (SEQ ID NO: 2);

Figure 3 provides nucleotide sequencing ladders for the von Willebrand's disease mutation region for normal (clear), carrier, and affected Scottish terriers, the sequences being obtained directly from PCR products derived from genomic DNAs in exon 4;

Figure 4 illustrates the results of a method of the present invention used to detect the Scottish terrier vWD mutation (SEQ ID NOS: 3-13);

Figure 5 shows the Scottish terrier pedigree, which in turn illustrates segregation of the mutant and normal vWF alleles;

Figure 6 is an illustration showing the splice site comparison between normal and mutant Doberman pinscher vWF alleles (SEQ ID NOS: 14-17);

Figure 7 is a photograph of a sequencing ladder showing the cryptic splice cite from the mutant allele (SEQ ID NO: 18);

Figure 8 is a photograph of an agarose gel showing representative results of the PCR-based diagnostic test;

Figure 9 is a histogram of genotypes versus reported vWF values;

Figure 10 is a photograph of a sequencing gel showing the mutation region between a vWD affected and a homozygous normal Shetland sheepdog (SEQ ID NOS: 19 and 20);

Figure 11 is a diagram illustrating the *Mwo I* diagnostic test for the Shetland sheepdog Type 3 vWD mutation (SEQ ID NOS: 21-25); and

Figure 12 is a photograph of an agarose gel showing the results of the diagnostic test for the Shetland sheepdog Type 3 vWD mutation.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The cDNA encoding canine von Willebrand Factor (vWF) has been sequenced, and is set forth in Figures 1A-1C and SEQ ID NO: 1. The deduced amino acid sequence is set forth in Figures 2A-2C and SEQ ID NO: 2. In one embodiment, the mutation of the normal vWF gene which causes von Willebrand's Disease (vWD) in Scottish terriers, a deletion at codon 88 of the normal gene resulting in a frameshift, is provided. In another embodiment, a splice junction mutation at nucleotide position 7639 of the normal gene, which causes vWD in Doberman pinschers, Manchester terriers and Poodles, is provided. In yet another embodiment, a single base deletion at nucleotide position 937 of the normal gene, causing vWD in Shetland sheepdogs, is provided. The nucleic acid sequences of the present invention may be used in methods for detecting homozygous and heterozygous carriers of the defective vWF gene.

In a preferred method of detecting the presence of the von Willebrand allele in canines, DNA samples are first collected by relatively noninvasive techniques, *i.e.*, DNA samples are obtained with minimal penetration into body tissues of the animals to be tested. Common noninvasive tissue sample collection methods may be used and include withdrawing buccal cells via cheek swabs and withdrawing blood samples. Following isolation of the DNA by standard techniques, PCR is performed on the DNA utilizing pre-designed primers that produce enzyme restriction sites on those DNA samples that harbor the defective gene. Treatment of the amplified DNA with appropriate restriction enzymes such as *Bsi*E I thus allows one to analyze for the presence of the defective allele. One skilled in the art will appreciate that this method may be applied not only to Scottish terriers, Doberman pinschers, Shetland sheepdogs, Manchester terriers and Poodles, but to other breeds such as Dutch Kooikers, as well.

The presence of the von Willebrand allele in canines can also be detected utilizing ligation amplification reaction technology (LAR) known to those skilled in the art. LAR is a method analogous to PCR for DNA amplification wherein ligases are employed for elongation in place of polymerases used for PCR. Another alternate method for detecting the presence of the canine von Willebrand allele also known to those skilled in the art, is allele specific oligonucleotide hybridization, wherein an oligonucleotide of about 20 bp containing the contiguous nucleotides of the allele of interest is hybridized to the canine DNA.

The present invention provides breeders with an accurate, definitive test whereby the undesired, defective vWF gene may be eliminated from breeding lines. The current tests used by breeders are protein-based, and as noted previously, the primary difficulty with this type of test is the variability of results due to a variety of factors. The ultimate result of such variability is that an inordinate number of animals fall into an ambiguous grouping whereby carriers and noncarriers cannot be reliably distinguished. The present invention obviates the inherent limitations of protein-based tests by detecting the genetic mutation which causes vWD. As described in the Specific Examples, the methods of the present invention provide an accurate test for distinguishing noncarriers, homozygous carriers and heterozygous carriers of the defective vWF gene.

It will be appreciated that because the vWF cDNA of the present invention is substantially homologous to vWF cDNA throughout the canine species, the nucleic acid sequences of the present invention may be used to detect DNA mutations in other breeds as well. In addition, the canine vWF sequence presented herein potentially in combination with the established human sequence (Genbank Accession No. X04385, Bonthron, D. et al., *Nucleic Acids Res.* 14:7125-7128 (1986); Mancuso, D.J. et al., *Biochemistry* 30:253-269 (1989); Meyer, D. et al., *Throm Haemostasis* 70:99-104 (1993)), may be used to facilitate sequencing of the vWF gene and genetic defects causing vWD, in other mammalian species *e.g.,* by using cross-species PCR methods known by those skilled in the art.

It is also within the contemplation of this invention that the isolated and purified nucleic acid sequences of the present invention be incorporated into an appropriate recombinant expression vector, *e.g.*, viral or plasmid, which is capable of transforming an appropriate host cell, either eukaryotic (*e.g.*, mammalian) or prokaryotic (*e.g.*, *E. coli*). Such DNA may involve alternate nucleic acid forms, such as cDNA, gDNA, and DNA prepared by partial or total chemical synthesis. The DNA may also be

accompanied by additional regulatory elements, such as promoters, operators and regulators, which are necessary and/or may enhance the expression of the vWF gene product. In this way, cells may be induced to over-express the vWF gene, thereby generating desired amounts of the target vWF protein. It is further contemplated that the canine vWF polypeptide sequence of the present invention may be utilized to manufacture canine vWF using standard synthetic methods.

One skilled in the art will appreciate that the defective protein encoded by the defective vWF gene of the present invention may also be of use in formulating a complementary diagnostic test for canine vWD that may provide further data in establishing the presence of the defective allele. Thus, production of the defective vWF polypeptide, either through expression in transformed host cells as described above for the active vWF polypeptide or through chemical synthesis, is also contemplated by the present invention.

The term "gene" as to referred herein means a nucleic acid which encodes a protein product. The term "nucleic acid" refers to a linear array of nucleotides and nucleosides, such as genomic DNA, cDNA and DNA prepared by partial or total chemical synthesis from nucleotides. The term "encoding" means that the nucleic acid may be transcribed and translated into the desired polypeptide. "Polypeptide" refers to amino acid sequences which comprise both full-length proteins and fragments thereof. "Mutation" as referred to herein includes any alteration in a nucleic acid sequence including, but not limited to, deletions, substitutions and additions.

As referred to herein, the term "capable of hybridizing under high stringency conditions" means annealing a strand of DNA complementary to the DNA of interest under highly stringent conditions. Likewise, "capable of hybridizing under low stringency conditions" refers to annealing a strand of DNA complementary to the DNA of interest under low stringency conditions. In the present invention, hybridizing under either high or low stringency conditions would involve hybridizing a nucleic acid sequence (*e.g.*, the complementary sequence to SEQ ID NO: 1 or portion thereof), with a second target nucleic acid sequence. "High stringency conditions" for the annealing process may involve, for example, high temperature and/or low salt content, which disfavor hydrogen bonding contacts among mismatched base pairs. "Low stringency conditions" would involve lower temperature, and/or higher salt concentration than that of high stringency conditions. Such conditions allow for two DNA strands to anneal if substantial, though not near complete complementarity exists between the two strands, as is the case among DNA strands that code for the

same protein but differ in sequence due to the degeneracy of the genetic code. Appropriate stringency conditions which promote DNA hybridization, for example, 6X SSC at about 45 °C, followed by a wash of 2X SSC at 50 °C are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, NY (1989), 6.31-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2X SSC at 50 °C to a high stringency of about 0.2X SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency at room temperature, about 22 °C, to high stringency conditions, at about 65 °C. Other stringency parameters are described in Maniatis, T., et al., *Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press*, Cold Spring NY, (1982), at pp. 387-389; see also Sambrook J. et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Volume 2, Cold Spring Harbor Laboratory Press, Cold Spring, NY at pp. 8.46-8.47 (1989).

SPECIFIC EXAMPLE 1 - SCOTTISH TERRIERS

Materials And Methods

Isolation of RNA. The source of the RNA was a uterus from a Scottish Terrier affected with vWD (factor level < 0.1% and a clinical bleeder), that was surgically removed because of infection. Spleen tissue was obtained from a Doberman pinscher affected with vWD that died from dilated cardiomyopathy (factor level 7% and a clinical bleeder). Total RNA was extracted from the tissues using Trizol (Life Technologies, Gaithersburg, MD). The integrity of the RNA was assessed by agarose gel electrophoresis.

Design of PCR primer sets. Primers were designed to a few regions of the gene, where sequences from two species were available (Lavergne, J.M. et al., *Biochem Biophys Res Commun* 194:1019-1024 (1993); Bakhshi, M.R. et al., *Biochem Biophys Acta* 1132:325-328 (1992)). These primers were designed using rules for cross-species' amplifications (Venta et al., "Gene-Specific Universal Mammalian Sequence-Tagged Sites: Application To The Canine Genome" *Biochem. Genet.* 34:321-341 (1996)). Most of the primers had to be designed to other regions of the gene using the human sequence alone (Mancuso, D.J. et al., *Biochemistry* 30:253-269 (1991)). Good amplification conditions were determined by using human and canine genomic DNAs.

Reverse Transcriptase-PCR. Total RNA was reverse transcribed using random primers (Bergenhem, N.C.H. et al., *PNAS (USA)* 89:8789-8802 (1992)). The cDNA was amplified using the primer sets shown to work on canine genomic DNA.

DNA Sequence Analysis. Amplification products of the predicted sizes were isolated from agarose gels by adsorption onto silica gel particles using the manufacturer's method (Qiagen, Chatsworth, CA). Sequences were determined using ^{33}P-5′ end-labeled primers and a cycle sequencing kit (United States Biochemical Corp., Cleveland, OH). The sequences of the 5′ and 3′ untranslated regions were determined after amplification using Marathon™ RACE kits (Clontech, Palo Alto, CA). Sequences were aligned using the Eugene software analysis package (Lark Technologies, Houston, TX). The sequence of the canine intron four was determined from PCR-amplified genomic DNA.

Design of a Diagnostic Test. PCR mutagenesis was used to create diagnostic and control *Bsi*E I and *Sau*96 I restriction enzyme sites for the test. Amplification conditions for the test are: 94°C, 1 min, 61°C, 1 min, and 72°C, 1 min, for 50 cycles using cheek swab DNA (Richards, B. et al., *Human Molecular Genetics* 2:159-163 (1992)).

Population Survey. DNA was collected from 87 Scottish terriers from 16 pedigrees. DNA was isolated either from blood using standard procedures (Sambrook, J. et al., Cold Harbor Spring Lab, Cold Harbor Spring NY, 2nd Edition, (1989)) or by cheek swab samples (Richards, B. et al., *Human Molecular Genetics* 2:159-163 (1992)). The genetic status of each animal in the survey was determined using the *Bsi*E I test described above.

Results

Comparison of the canine and human sequences. The alignment of the canine and human prepro-von Willebrand Factor amino acid sequences is shown in Figures 2A-2C (SEQ ID NO: 2). The location of the Scottish terrier vWD mutation is indicated by the "*". Potential N-glycosylation sites are shown in bold type. The known and postulated integrin binding sites are boxed. Amino acid numbers are shown on the right side of the figure. The human sequence is derived from Genbank accession number X04385.

Overall, 85.1% sequence identity is seen between the prepro-vWF sequences. The pro-region is slightly less conserved than the mature protein (81.4% vs. 87.5%). There were no other noteworthy percentage sequence identity differences seen in other regions of the gene, or between the known repeats contained within the gene (data not shown). Fourteen potential N-linked glycosylation sites are present in the canine sequence, all of which correspond to similar sites contained within the human sequence. The two integrin binding sites identified in the human vWF protein

sequence (Lankhof, H. et al., *Blood* 86:1035-1042 (1995)) are conserved in the canine sequence as well (Figures 2A-2C; SEQ ID NO: 2). The 5′ and 3′ untranslated regions have diverged to a greater extent than the coding region (data not shown), comparable to that found between the human and bovine sequences derived for the 5′ flanking region (Janel, N. et al., *Gene* 167:291-295 (1995)). Additional insights into the structure and function of the von Willebrand factor can be gained by comparison of the complete human sequence (Genbank Accession No. XO4385; Bonthron, D. et al., *Nucleic Acids Res.* 14:7125-7128 (1986); Mancuso, D.J. et al., *Biochemistry* 30:253-269 (1989); Meyer, D. et al., *Throm Haemostasis* 70:99-104 (1993)) and the complete canine sequence reported here.

The sequence for most of exon 28 was determined (Mancuso, D.J. et al., *Thromb Haemost* 69:980 (1993); Porter, C.A. et al., *Mol Phylogenet Evol* 5:89-101 (1996)). All three sequences are in complete agreement, although two silent variants have been found in other breeds (Table 1, exon 28). Partial sequences of exons 40 and 41 (cDNA nucleotide numbers 6923 to 7155, from the initiation codon) were also determined as part of the development of a polymorphic simple tandem repeat genetic marker (Shibuya, H. et al., *Anim Genet* 24:122 (1994)). There is a single nucleotide sequence difference between this sequence ("T") and the sequence of the present invention, ("C") at nucleotide position 6928.

Scottish Terrier vWD mutation. Figure 3 shows nucleotide sequencing ladders for the vWD mutation region for normal (clear), carrier, and affected Scottish terriers. The sequences were obtained directly from PCR products derived from genomic DNAs in exon 4. The arrowheads show the location of the C nucleotide that is deleted in the disease-causing allele. Note that in the carrier ladder each base above the point of the mutation has a doublet appearance, as predicted for deletion mutations. The factor levels reported for these animals were: Normal, 54%; Carrier, 34%; Affected, <0.1%.

As a result of the deletion, a frameshift mutation at codon 88 leads to a new stop codon 103 bases downstream. The resulting severely truncated protein of 119 amino acids does not include any of the mature vWF region. The identity of the base in the normal allele was determined from an unaffected dog.

Development of a diagnostic test. A PCR primer was designed to produce a *Bsi*E I site in the mutant allele but not in the normal allele (Figure 4; SEQ ID NOS 3 and 10). The position of the deleted nucleotide is indicated by an asterisk. The altered nucleotides in each primer are underlined. The normal and mutant allele can

also be distinguished using *Sau*96 I. The naturally occurring *Sau*96 I sites are shown by double underlines. The highly conserved donor and acceptor dinucleotide splice sequences are shown in bold type.

In order to ensure that the restriction enzyme cut the amplified DNA to completion, an internal control restriction site common to both alleles was designed into the non-diagnostic primer. The test was verified by digestion of the DNA from animals that were affected, obligate carriers, or normal (based on high factor levels [greater than 100% of normal] obtained from commonly used testing labs and reported by the owners, and also using breeds in which Type 3 vWD has not been observed). The expected results were obtained (*e.g.*, Figure 5). Five vWD-affected animals from a colony founded from Scottish terriers (Brinkhous, K.M. et al., *Ann. New York Acad. Sci.* 370:191-203 (1981)) were also shown to be homozygous for this mutation. An additional unaffected animal from this same colony was found to be clear.

It would still be possible to misinterpret the results of the test if restriction enzyme digestion was not complete, and if the rates of cleavage of the control and diagnostic sites were vastly different. The rates of cleavage of the two *Bsi*E I sites were thus examined by partially digesting the PCR products and running them on capillary electrophoresis. The rates were found to be very nearly equal (the diagnostic site is cut 12% faster than the control site).

The mutagenesis primer was also designed to produce a *Sau*96 I site into the normal allele but not the mutant allele. This is the reverse relationship compared to the *Bsi*E I-dependent test, with respect to which allele is cut. Natural internal *Sau*96 I sites serve as digestion control sites (shown in Figure 4). The test using this enzyme produced identical genotypic results compared to the *Bsi*E I for all animals examined (data not shown).

Mendelian inheritance. One test often used to verify the correct identification of a mutant allele is its inheritance according to Mendel's law of segregation. Three pedigrees were examined in which the normal and mutant alleles were segregating, as shown in Figure 5. Exon four of the vWF gene was PCR-amplified from genomic DNA. The PCR products were examined for the presence of the normal and mutant vWF alleles by agarose gel electrophoresis after digestion with *Bsi*E I (see Figure 5). The affected animals are homozygous for the mutant allele (229 bp; lanes 3 and 5). The other animals in this pedigree are heterozygotes (251 bp and 229 bp; lanes 1, 2, 4, and 6), including the obligate carrier parents.

Table 1 - Differences Between Scottie And Doberman Pinscher Protein And Nucleotide von Willebrand Factor Sequences With Comparison To The Human Sequences

		Amino Acid			Codon		
Exon	A.A.[1]	Human	Scottie	Doberman	Human	Scottie	Doberman
5' UT[2]	nuc - 35[3]	N/A[4]	N/A	N/A	N/A	A	G
4	85	S	S/F.Shift[5]	S	TCC	TCC/TC_	TCC
5	173	M	R	K	ATG	AGG	AAG
11	422	S	T	T	TCC	ACA	ACC
21	898	C	C	C	TGC	TGT	TGC
21	905	F	F	L	TTT	TTC	TTA
24	1041	S	S	S	TCA	TCA	TCG
24	1042	S	S	S	TCC	TCC	TCA
28	1333	D	D	E	GAC	GAC	GAG
28	1349	Y	Y	Y	TAT	TAT	TAC*
42	2381	P	L	P	CCC	CTG	CCG
43	2479[6]	S	S	S	TCG	TCG	TCA
45	2555	P	P	P	CCC	CCC	CCG
47	2591	P	P	P	CCC	CCT	CCC
49	2672	D	D	D	GAT	GAT	GAC
51	2744	E	E	E	GAG	GAG	GAA

[1]Amino acid residue position
[2]Untranslated region
[3]Nucleotide position
[4]Not Applicable
[5]Frameshift mutation
[6]Splice site mutation for Doberman pinscher, Manchester terrier and Poodle
Boxed residues show amino acid differences between breeds
*This site has been shown to be polymorphic in some breeds
The mature VWF protein begins in exon 18

The alleles, as typed by both the *Bsi*E I and *Sau*96 I tests, showed no inconsistencies with Mendelian inheritance. One of these pedigrees included two affected animals, two phenotypically normal siblings, and the obligate carrier parents. The two parents were found to be heterozygous by the test, the two affected animals were found to be homozygous for the mutant allele, and the normal siblings were found to be heterozygotes.

Population survey for the mutation. Cheek swabs or blood samples were collected from 87 animals in order to determine the incidence of carriers in the U.S.

Scottish terrier population. Although an attempt was made to make the sample as random as possible, these dogs were found to come from 16 pedigrees, several of which are more distantly interconnected. This is due to some ascertainment bias, based on ownership (as opposed to phenotypic ascertainment bias). In these 87 animals, 4 affected and 15 carrier animals were found.

Discussion

These results establish that the single base deletion found in exon four of the vWF gene causes vWD in the Scottish terrier breed. The protein produced from the mutant allele is extremely short and does not include any of the mature vWF protein. Four Scottish terriers known to be affected with the disease are homozygous for the mutation. Five other mixed-breed dogs descended from Scottish terriers, and affected with vWD, are also homozygous for the mutation. No normal animals are homozygous for the mutation. Unaffected obligate carriers are always heterozygous for the mutation.

The gene frequency, as determined from the population survey, appears to be around 0.13 resulting in a heterozygote frequency of about 23% and expected frequency of affected animals of about 2%. Although the sample size is relatively small and somewhat biased, these data are in general agreement with the protein-based surveys (Stokol, T. et al., *Res Vet Sci* 59:152-155 (1995); Brooks, M., *Probl In Vet Med* 4:636-646 (1992)), in that the allele frequency is substantial.

All data collected thus far indicate that this mutation may account for essentially all of the von Willebrand's disease found in Scottish terriers. This result is consistent with the results found for other genetic diseases, defined at the molecular level, in various domestic animals (Shuster, D.E. et al., *PNAS (USA)* 89:9225-9229 (1992); Rudolph, J.A. et al., *Nat Genet* 2:144-147 (1992); O'Brien, P.J. et al., *JAVMA* 203:842-851 (1993)). A likely explanation may be found in the pronounced founder effect that occurs in domestic animals, compared to most human and wild animal populations.

Published data using the protein-based factor assays have shown that, at least in several instances, obligate carriers have had factor levels that would lead to a diagnosis of "clear" of the disease allele. For example, in one study an obligate carrier had a factor level of 78% (Johnson, G.S. et al., *JAVMA* 176:1261-1263 (1980)). In another study, at least some of the obligate carriers had factor levels of 65% or greater (Brinkhous, K.M. et al., *Ann. New York Acad. Sci.* 370:191-203 (1981)). In addition, the number of animals that fall into an equivocal range can be

substantial. In one study, 19% of Scottish terriers fell in this range (50-65% of the normal vWF antigen level) (Stokol, T. et al., *Res Vet Sci* 59:152-155 (1995)). Thus, although the protein-based tests have been useful, the certainty of the DNA-based test described herein should relieve the necessity of repeated testing and the variability associated with the protein-based assays.

The mutation is present in the pre-vWF part of the molecule. This part of the molecule is processed off prior to delivery of the mature protein into the plasma. This pre-portion of the molecule is important for the assembly of the mature vWF protein (Verwiej, L. et al., *EBMO J* 6:2885-2890 (1987); Wise, R.J. et al., *Cell* 52:229-236 (1988)). With the Scottish terrier frameshift vWD mutation, neither this pre-portion nor any of the mature factor is ever produced, in keeping with the fact that no factor has ever been detected in the blood of affected dogs.

The determination of the complete canine vWF cDNA sequence will have an impact upon the development of carrier tests for other breeds and other species as well. Currently, Shetland sheepdogs (see Specific Example 3) and Dutch Kooikers are known to have a significant amount of Type 3 vWD (Brooks, M. et al., *JAVMA* 200:1123-1127 (1992); Slappendel, R.J., *Vet-Q* 17:S21-S22 (1995)). Type 3 vWD has occasionally be seen in other breeds as well (*e.g.*, Johnson, G.S. et al., *JAVMA* 176:1261-1263 (1980)). All Type 3 vWD mutations described in humans to date have been found within the vWF gene itself. The availability of the canine sequence will make it easier to find the mutations in these breeds. In addition, at least some Type 1 mutations have been found within the human vWF gene, and thus Type 1 mutations may also be found within the vWF gene for breeds affected with that form of the disease. The availability of two divergent mammalian vWF cDNA sequences will also make it much easier to sequence the gene from other mammalian species using cross-species PCR methods (e.g., Venta et al., *Biochem. Genet.* 34:321-341 (1996)).

The test described herein for the detection of the mutation in Scottish terriers may be performed on small amounts of DNA from any tissue. The tissues that are the least invasive to obtain are blood and buccal cells. For maximum convenience, a cheek swab as a source of DNA is preferred.

SPECIFIC EXAMPLE 2 - DOBERMAN PINSCHER

Materials and Methods

RT-PCR and DNA Sequence Analysis. RNA was isolated by using Trizol (Life Technologies, Gaitherburg, MD) from the spleen of a Doberman pinscher that was affected with vWD (factor value of 7% of normal) and that had died from dilated

cardiomyopathy. RT-PCR was performed as previously described using primers to the canine vWF cDNA. Most PCR products were determined directly using a cycle sequencing kit (Amersham Corp, Chicago, IL). A minor band containing the four base deletion (see Results) was subcloned into a plasmid vector prior to sequence analysis. The five kb intron 43 was amplified using a commercially available kit for long PCR (Boehringer-Mannheim, Indianapolis, IN). The cycling times and temperatures were as follows: initial denaturation, 93°C, 2 min; 10 cycles of 93°C, 15 sec, 62°C, 30 sec, 68°C, 4 min; 20 cycles of 93°C, 15 sec, 62°C, 30 sec, 68°C, 4 min with 20 additional sec per cycle. This was followed by a final extension at 68°C for 7 min. The sequences of the primers used were: exon 43 (sense primer), 5'-TCTACCCTGTGGGCCAGTTC-3' (SEQ ID NO: 26), and exon 44 (antisense primer), 5'-GACCACCTCACAGGCAGAT-3' (SEQ ID NO: 27).

PCR-Based Mutation Test. PCR mutagenesis was used to create an *Msp* I site in the normal allele but not in the mutant allele. An internal *Msp* I digestion control site was also created by PCR mutagenesis within the anti-sense primer, whose target is within intron 43. The control site is contained within the amplification products of both alleles. The sequences of the primers are: diagnostic (sense) primer, 5'-CTGTGAGGACAACTGCCTGCC-3' (SEQ ID NO: 28); and common (anti-sense) primer, 5'-TGGCCCTGAACCGGAAATTACTCAAG-3' (SEQ ID NO: 29) (the altered bases within each primer are underlined). A 'touchdown' PCR protocol was used for the amplification. The amplification conditions are: 94°C, 30 sec, 63 to 55°C, 40 sec, and 72°C, 50 sec, for the first 8 cycles, with the annealing temperature dropping one degree per cycle. Twenty-eight additional cycles were run, with the annealing temperature held at 55°C. The DNA was digested with *Msp* I after PCR amplification.

Population Survey. Owners who participated in a population survey supplied cheek swabs from their dogs for genotype analysis. Richards, B. et al., *Hum. Mol. Genet.* 2:159 (1992). A number of these dogs had associated vWF values that were determined by various testing laboratories that provide this service to breeders.

Results

During the sequence analysis of the vWF mRNA from an affected Doberman pinscher, a significant nucleotide difference from the Scottish terrier sequence was discovered. This change was found at the last base of exon 43 (nucleotide 7437 from the initiation codon, at amino acid position Ser 2479; G in Scotties, A in the affected Doberman) (Table 1). Although this is a silent amino acid change, it causes the

splice junction to be less similar to the mammalian splice junction consensus. Nakai, K. et al., *Gene* 141:171 (1994); Krawcsak, M. et al., *Genet.* 90:41 (1992). Just upstream of the normal splice junction is another sequence that also has significant similarity to the consensus, which is increased by the A at nucleotide position 7437 (Figure 6; SEQ ID NOS: 14-17). The A at the end of exon 43 could cause the normal splice junction to be used less frequently, and that the upstream cryptic splice site becomes the one predominantly used. Comparison of the splice sites by a devised statistical method (Shapiro, M.B. et al., *Nucleic Acids Res.* 15:7155 (1987)) gave the following scores: normal splice position with the wild-type allele (G at 7536), 83.9; cryptic splice site with the wild-type allele, 60.6; normal splice position with the mutant allele (A at 7437), 72.2; cryptic splice site with the mutant allele, 70.5. Higher scores represent a greater likelihood of splicing potential. The scores for the normal and cryptic splice sites are quite different with the wild-type allele, but are very close with the mutant allele. These results support the probability of a decreased likelihood for splicing at the normal site, and an increased potential for splicing at the cryptic site with the mutant allele.

A faint RT-PCR band just below the major band from which the variant nucleotide had been detected was observed. This minor band was missing the four bases at the end of exon 43 as confirmed by sequence analysis (Figure 7; SEQ ID NO: 18). The position of the four deleted bases is shown on the right side of Figure 7 (SEQ ID NO: 18).

A PCR-based test was developed to detect the nucleotide difference in genomic DNA as described herein in Materials and Methods. The results of the test for several animals with a spectrum of factor values yield a significant correlation between genotype and factor value as shown in Figure 8. Lane 1 contains a 50 bp ladder as a size marker. The uncut PCR product is 135 bp (lane 8). Both alleles contain a common Msp I restriction site that serves as an internal digestion control. The mutant (A) and normal (G) alleles are represented by the 123 bp and 102 bp bands, respectively. Reported factor levels and deduced genotypic status for dogs represented in the additional lanes are as follows: 2, 12 %, affected (AA); 3, 8 %, affected (AA); 4, 39 %, carrier (AG); 5, 68 %, carrier (AG); 6, 125 %, homozygous normal (GG); 7, 136 %, homozygous normal (GG). A survey of 21 randomly ascertained animals with associated factor values showed a strong correlation between genotype and factor level as presented in the histogram of Figure 9. The shaded boxes indicate predicted genotypes based on factor levels that are not

consistent with the genotypes deduced from the PCR-based diagnostic test. Larger factor value-only surveys (Johnson et al., *Vet. Clin. North Am. Small Anim. Pract.* 18:195-229 (1988); Moser et al., *Am. J. Vet. Res.* 57:1288-1293 (1996); Stokol et al., *Aust. Vet. J.* 72:257-262 (1996)) indicate substantial overlap between genotypes based upon the protein-based methods. A larger survey on 67 additional Dobermans contained in 10 independently ascertained pedigrees was performed to obtain an estimate of the mutant allele frequency within the breed. Of the total of 88 animals, 40 were AA, 35 were AG, and 13 were GG. From these results, the A allele frequency was estimated to be 0.64.

Discussion

The splice junction mutation at the end of exon 43 is the cause of recessive Type 1 vWD found within the Doberman pinscher breed. The mutation decreases the similarity between the normal splice junction and the mammalian consensus while at the same time increasing the similarity of the cryptic splice site found just upstream of the normal splice site (Figure 6; SEQ ID NOS: 14-17). The calculated Shapiro-Senapathy splice site values (Shapiro, M.B. et al., *Nucleic Acids Res.* 15:7155 (1987)) are very similar for the normal and cryptic splice sites when an A is present at nucleotide position 7536. The Shapiro-Senapathy calculation is probably not completely accurate in determining the relative amount of splicing that can occur between different sites. Therefore, it is not inconsistent to find that the cryptic splice site is used more often than the normal site, in the mutant allele.

The sequence of the minor amplification product seen just below the main amplification band exactly matches that predicted by the use of the cryptic splice site (Figure 7; SEQ ID NO: 18). The fact that there is less cryptically spliced mRNA than normally spliced mRNA present in the cytoplasm can be explained by the relative instability of the cryptically spliced message. The cryptically spliced mRNA produces a shift in the translational reading frame, resulting in the formation of a premature stop codon. It is well known that mRNAs that produce truncated proteins are unstable, perhaps because ribosomes do not remain attached to the message to protect it from degradation by intracellular RNases or because of the incomplete assembly of splicosomes on mutant splice sites. Maquat, L.E., *Am J Hum Genet* 59:279 (1996). The average amount of vWF protein present in affected animals is roughly 10% of the normal canine value. Thus, each mutant allele should produce about 5% of the normal amount of vWF mRNA and protein. From this, it can be predicted that the average heterozygous Doberman should produce 55% of the average canine vWF

value. The vWF mRNA estimated in affected animals has been shown to be roughly 20% of normal by densitometry scans of northern blots. Meinkoth, J.H. et al., *Am. J. Vet. Res.* 56:1577 (1995). This mRNA is predicted to consist primarily of the correctly spliced transcript.

The mutation has been shown to be linked to the vWF locus (Figure 9 and Holmes, N.G. et al., *J. Small An. Prac* 37:307 (1996). Most human Type 1 vWD, in which there is a true clinical bleeding problem, appears to be inherited in a dominant, incompletely penetrant fashion. Ginsburg, D. et al., *Blood* 79:2507 (1992). Although a few Type 1 mutations have been found within the vWF locus (see, *e.g.*, Siguret, V. et al., *Hum. Genet.* 93:95 (1994); Eikenboom, J.C.J. et al., *Blood* 88:2433 (1996)), it has been argued that another locus or loci may also cause some Type 1 vWD. Ginsburg, D. et al., *Blood* 79:2507 (1992). In fact, one murine Type 1 vWD has been mapped to locus that is not linked to the vWF gene. Nichols, W.C. et al., *Blood* 83:3225 (1994). The data show that a least a proportion of Type 1 vWD in humans might also be caused by the exon 43 mutation, or other leaky splice junction mutations. The mode of inheritance for this type of mutation is recessive, but it might appear to be dominant in certain situations, such as that of the Doberman pinscher. The number of splice site mutations of the type described herein are significantly below the number that would be predicted to occur, suggesting that these types of mutations are more difficult to detect or have been overlooked in the past. Krawcsak, M. et al., *Hum. Genet.* 90:41 (1992). This might be because they produce a less severe phenotype than other types of mutations that cause a complete loss of function.

SPECIFIC EXAMPLE 3 - SHETLAND SHEEPDOG

Total DNA was isolated from material obtained from a spay of an affected Shetland sheepdog (Sheltie). This animal had been tested for the vWF antigen, and was reported to have a 0% value by a laboratory skilled in this testing (Diagnostic Laboratory, Comparative Hematology Section, College of Veterinary Medicine, Cornell University). The owner had decided to have the spay done after obtaining this result, and donated the removed tissues. The entire RT-PCR coding region of this mutant gene was sequenced as described in Specific Example 1, to identify the mutation that causes vWD. A mutation was found in the vWF gene that appears to be responsible for most or all of the type 3 vWD found in the Sheltie breed. A deletion of a single T was found at nucleotide position 735 of the encoding region (Figure 10; SEQ ID NOS: 19 and 20). The arrows in Figure 10 indicate the series of T nucleotides in

which one T has been deleted in the DNA of the affected animal compared to the normal animal. This deletion, present in the equivalent of human exon 7, would cause a shift in the reading from of the vWF-encoding region, and result in a severely truncated protein. A diagnostic test was designed to detect this mutation (Figure 11; SEQ ID NOS: 21-25). The deletion causes the creation of an *Mwo* I restriction site and thus, the *Mwo* site is found in the mutant allele, but not in the normal allele. The sequence shown in Figure 11 (SEQ ID NOS: 21 and 22) is that of the canine gene that corresponds to the human vWF exon 7. The single letter code for amino acids is shown above the nucleotide sequence and the primer sequences are shown below the gene sequence. The *Mwo* I sites are also indicated. An internal digestion control site is present in the non-diagnostic primer region. Reagent concentrations for this test were: 100 μM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.05 to 0.1 μg target DNA, 15 μM of each primer (SEQ ID NOS: 23 and 25), and 0.025 U Taq DNA polymerase. Cycling conditions were: 94°C, 4 min, one cycle, followed by 50 cycles of 94°C, 30 sec, 63°C, 40 sec, and 72°C, 40 sec. The relatively low Taq concentration (compared to generally accepted conditions) with the high number of cycles prevents the amplification of non-specific PCR bands. One microliter of *Mwo* I restriction enzyme (New England Biolabs, Inc.) and 2 μl of 50 mM $MgCl_2$ were added directly to the PCR reaction after amplification, and incubated at 60°C for 1 hr. Digestion products were then observed after gel electrophoresis on a 1.5% agarose gel and the results shown in Figure 12. Lanes 1 and 17 show a one hundred bp ladder. Lanes 2-6 show the results from an affected animal, lanes 7-11 show the results from a carrier animal, and lanes 12-16 show the results from a homozygous normal animal. Lane 18 shows an undigested control PCR product. The duplicate samples demonstrate the reproducibility of the test. Numbers on the left side of the gel show the sizes of the standard bands, and numbers on the right side of the gel show the sizes of the uncut product (U), the normal allele (N), and the two bands for the mutant allele (M).

A survey of Shelties was conducted to determine the frequency of the mutation within the U.S. population. Of a total of 103 animals, 14 were carriers, giving a carrier frequency of 13.6%. This frequency is less than the value of 28% reported for the breed in 1988 for 730 animals when using the factor antigen test. Brooks, M. et al., *J. Am. Vet. Med. Assoc.* 200:1123-1127 (1992). One third of these carriers are thought to be due to Type 1 vWD also present in the breed. Still, the value of 13.6% would be lower than the calculated value of 18.7% from the antigen test. This

difference could be due to either ascertainment biases in either study, a true decrease in the frequency of the disease in this breed, one or more additional Type 3 mutations in the breed, or a combination of these possibilities. Whatever the reason for the difference, most or all of the Type 3 disease in the Sheltie is probably caused by this one mutation. This is based on the understanding of the importance of the Founder effect (or populate sire effect) on the increase in the frequency of specific genetic diseases in purebred populations of domestic animals. A 17 member pedigree of Shelties, in which the mutation was segregating was tested for normal Mendelian inheritance of the allele. There were no differences from what would be expected under co-dominant inheritance of the two alleles.

SPECIFIC EXAMPLE 4

In an effort to find mutations that cause vWD in other canine breeds, affected animals were surveyed, as diagnosed by low levels of vWF antigen, for the three mutations set forth herein. In the case of the Manchester terrier breed, it was found that at least a portion of the affected animals had the identical mutation that causes vWD in the Doberman pinscher. The test described *supra* for the Doberman pinscher was utilized to test an affected Manchester terrier, plus several related animals. The affected animal was found to be homozygous for the mutant allele (Table 2). In addition, several animals who had vWF values in the carrier range were found to be carriers at the genotypic level.

Table 2

Manchester terrier vWF values vs. DNA genotype

Dog	vWF value[a]	Genotype[b]
MT1	200%	normal
MT2	76%	normal
MT3	42%	carrier
MT4	19%	carrier
MT5	NT	carrier
MT6	NT	carrier
MT7	10%	affected

[a]Factor values as reported from a testing lab (Cornell CVM, Hematology Lab).
[b]Genotype for the leaky splice mutation originally found in the Doberman pinscher.

SPECIFIC EXAMPLE 5

In an effort to locate mutations that cause vWD in other canine breeds, affected animals as diagnosed by low levels of vWF antigen, were surveyed for the three mutations set forth herein. The test described *supra* for the Doberman pinscher was utilized and, in the case of the Poodle breed, it was found that the affected animals had the identical mutation that causes vWD in the Doberman pinscher. The affected animals were found to be homozygous for the mutant allele. In addition, several animals who had vWF values in the carrier range were found to be carriers at the genotypic level.

The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention.

All patents and other publications cited herein are expressly incorporated by reference.

WE CLAIM:

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding mutated canine von Willebrand Factor polypeptide which causes canine von Willebrand's disease, wherein the nucleotide sequence is capable of hybridizing under high stringency conditions to the complementary sequence of the sequence of SEQ ID NO. 1 having a mutation at nucleotide 7639.

2. A vector comprising the nucleic acid molecule of Claim 1.

3. A cell comprising the vector of Claim 2.

4. The isolated nucleic acid molecule of Claim 1, wherein the mutation at nucleotide 7639 is a substitution.

5. An isolated nucleic acid molecule comprising a nucleotide sequence encoding mutated canine von Willebrand Factor polypeptide which causes canine von Willebrand's disease, wherein the nucleotide sequence is capable of hybridizing under high stringency conditions to the complementary sequence of the sequence of SEQ ID NO. 1 having a deletion at nucleotide 937.

6. A vector comprising the nucleic acid molecule of Claim 5.

7. A cell comprising the vector of Claim 6.

8. A method of detecting a canine von Willebrand Factor gene in a sample comprising the steps of:

a) contacting the sample with an oligonucleotide comprising contiguous nucleic acids of the nucleotide sequence of SEQ ID NO. 1 having a mutation at nucleotide 7639, and capable of specifically hybridizing with the canine von Willebrand Factor gene, under conditions favorable for hybridization of the oligonucleotide to any complementary sequences of nucleic acid in the sample; and

b) detecting hybridization, thereby detecting a canine von Willebrand Factor gene.

9. The method of Claim 8, further comprising the step of:

c) quantifying hybridization of the oligonucleotide to the complementary sequence.

10. The method of Claim 8, wherein the mutation at nucleotide 7639 is a substitution.

11. A method of detecting a canine von Willebrand Factor gene in a sample comprising the steps of:

a) contacting the sample with an oligonucleotide comprising contiguous nucleic acids of the nucleotide sequence of SEQ ID NO. 1 having a deletion at nucleotide 937, and capable of specifically hybridizing with the canine von Willebrand Factor gene, under conditions favorable for hybridization of the oligonucleotide to any complementary sequences of nucleic acid in the sample; and

b) detecting hybridization, thereby detecting a canine von Willebrand Factor gene.

12. The method of Claim 11, further comprising the step of:

c) quantifying hybridization of the oligonucleotide to the complementary sequence.

13. An assay kit for screening for a canine von Willebrand Factor gene comprising:

a) an oligonucleotide comprising contiguous nucleic acids of the nucleotide sequence of SEQ ID NO. 1 having a mutation at nucleotide 7639, and capable of hybridizing with the nucleotide sequence encoding canine von Willebrand Factor;

b) reagents for hybridization of the oligonucleotide to a complementary nucleic acid sequence; and

c) container means for a)-b).

14. The assay kit of Claim 13, wherein the mutation at nucleotide 7639 is a substitution.

15. An assay kit for screening for a canine von Willebrand Factor gene comprising:

a) an oligonucleotide comprising contiguous nucleic acids of the nucleotide sequence of SEQ ID NO. 1 having a deletion at nucleotide 937, and capable of hybridizing with the nucleotide sequence encoding canine von Willebrand Factor;

b) reagents for hybridization of the oligonucleotide to a complementary nucleic acid sequence; and

c) container means for a)-b).

16. An assay kit for screening for a canine von Willebrand Factor gene comprising:

a) an oligonucleotide comprising contiguous nucleic acids of the nucleotide sequence that is complementary to the sequence of SEQ ID NO. 1 having a mutation at nucleotide 7639, and capable of specifically hybridizing to the complementary nucleotide sequence;

b) reagents for hybridization of the oligonucleotide to a complementary nucleic acid sequence; and

c) container means for a)-b).

17. The assay kit of Claim 16, wherein the mutation at nucleotide 7639 is a substitution.

18. An assay kit for screening for a canine von Willebrand Factor gene comprising:

a) an oligonucleotide comprising contiguous nucleic acids of the nucleotide sequence that is complementary to the sequence of SEQ ID NO. 1 having a deletion at nucleotide 937, and capable of specifically hybridizing to the complementary nucleotide sequence;

b) reagents for hybridization of the oligonucleotide to a complementary nucleic acid sequence; and

c) container means for a)-b).

19. A method for detecting a mutated canine von Willebrand Factor gene in a canine DNA sample comprising the steps of:

a) amplifying the DNA sample by polymerase chain reaction to produce polymerase chain reaction products, wherein the polymerase chain reaction uses primers that produce a restriction site in a mutant allele but not in a normal allele, wherein the mutation in the mutant allele is a deletion at nucleotide 937 of the gene encoding canine von Willebrand Factor;

b) digesting the polymerase chain reaction products with a restriction enzyme specific to the restriction site of the restriction site primer to produce DNA fragments; and

c) detecting the DNA fragments, thereby detecting a mutated canine von Willebrand Factor gene.

20. The method of Claim 19, wherein the DNA fragments are detected by gel electrophoresis.

21. The method of Claim 19, wherein the primers have the sequence of SEQ ID NOS: 23 and 25.

22. The method of Claim 19, wherein the restriction enzyme is *Mwo* I.

23. A method for detecting a mutated canine von Willebrand Factor gene in a canine DNA sample comprising the steps of:

a) amplifying the DNA sample by polymerase chain reaction to produce polymerase chain reaction products, wherein the polymerase chain reaction uses primers that produce a restriction site in a mutant allele but not in a normal allele, wherein the mutation in the mutant allele is a substitution at nucleotide 7639 of the gene encoding canine von Willebrand Factor;

b) digesting the polymerase chain reaction products with a restriction enzyme specific to the restriction site of the restriction site primer to produce DNA fragments; and

c) detecting the DNA fragments, thereby detecting a mutated canine von Willebrand Factor gene.

24. The method of Claim 23, wherein the DNA fragments are detected by gel electrophoresis.

25. The method of Claim 23, wherein the primers have the sequence of SEQ ID NOS: 28 and 29.

26. The method of Claim 23, wherein the restriction enzyme is *Msp I*.

27. An oligonucleotide probe capable of detecting a mutation associated with canine von Willebrand's disease, wherein the mutation is a base substitution at nucleotide 7639 of the nucleotide sequence encoding canine von Willebrand Factor.

28. The oligonucleotide probe of Claim 27, wherein the substitution at nucleotide 7639 is adenine for guanine.

29. An oligonucleotide probe capable of detecting a mutation associated with canine von Willebrand's disease, wherein the mutation is a base deletion at nucleotide 937 of the nucleotide sequence encoding canine von Willebrand Factor.

ABSTRACT

The complete sequence of the canine von Willebrand Factor cDNA and deduced amino acid sequence is provided. The mutation which causes von Willebrand's Disease in Scottish Terriers, Doberman pinschers, Shetland sheepdogs, Manchester terriers and Poodles are also provided. Methods for detecting carriers of the defective vWF gene are also provided.

FIGURE 1A

```
   1 CATTAANAGG TCCTGGCTGG GAGCTTTTTT TTGGGACCAG CACTCCATGT TCAAGGGCAA
  61 ACAGGGGCCA ATTAGGATCA ATCTTTTTTC TTTCTTTTTT TAAAAAAAAA AATTCTTCCC
 121 ACTTTGCACA CGGACAGTAG TACATACCAG TAGCTCTCTG CGAGGACGGT GATCACTAAT
 181 CATTTCTCCT GCTTCGTGGC AGATGAGTCC TACCAGACTT GTGAGGGTGC TGCTGGCTCT
 241 GGCCCTCATC TTGCCAGGGA AACTTTGTAC AAAAGGGACT GTTGGAAGGT CATCGATGGC
 301 CCGATGTAGC CTTCTCGGAG GTGACTTCAT CAACACCTTT GATGAGAGCA TGTACAGCTT
 361 TGCGGGAGAT TGCAGTTACC TCCTGGCTGG GGACTGCCAG GAACACTCCA TCTCACTTAT
 421 CGGGGGTTTC CAAAATGACA AAAGAGTGAG CCTCTCCGTG TATCTCGGAG AATTTTTCGA
 481 CATTCATTTG TTTGTCAATG GTACCATGCT GCAGGGGACC CAAAGCATCT CCATGCCCTA
 541 CGCCTCCAAT GGGCTGTATC TAGAGGCCGA GGCTGGCTAC TACAAGCTGT CCAGTGAGGC
 601 CTACGGCTTT GTGGCCAGAA TTGATGGCAA TGGCAACTTT CAAGTCCTGC TGTCAGACAG
 661 ATACTTCAAC AAGACCTGTG GGCTGTGTGG CAACTTTAAT ATCTTTGCTG AGGATGACTT
 721 CAAGACTCAA GAAGGGACGT TGACTTCGGA CCCCTATGAC TTTGCCAACT CCTGGGCCCT
 781 GAGCAGTGGG GAACAACGGT GCAAACGGGT GTCCCCTCCC AGCAGCCCAT GCAATGTCTC
 841 CTCTGATGAA GTGCAGCAGG TCCTGTGGGA GCAGTGCCAG CTCCTGAAGA GTGCCTCGGT
 901 GTTTGCCCGC TGCCACCCGC TGGTGGACCC TGAGCCTTTT GTCGCCCTGT GTGAAAGGAC
 961 TCTGTGCACC TGTGTCCAGG GGATGGAGTG CCCTTGTGCG GTCCTCCTGG AGTACGCCCG
1021 GGCCTGTGCC CAGCAGGGGA TTGTCTTGTA CGGCTGGACC GACCACAGCG TCTGCCGACC
1081 AGCATGCCCT GCTGGCATGG AGTACAAGGA GTGCGTGTCC CCTTGCACCA GAACTTGCCA
1141 GAGCCTTCAT GTCAAAGAAG TGTGTCAGGA GCAATGTGTA GATGGCTGCA GCTGCCCCGA
1201 GGGCCAGCTC CTGGATGAAG GCCACTGCGT GGGAAGTGCT GAGTGTTCCT GTGTGCATGC
1261 TGGGCAACGG TACCCTCCGG GCGCCTCCCT CTTACAGGAC TGCCACACCT GCATTTGCCG
1321 AAATAGCCTG TGGATCTGCA GCAATGAAGA ATGCCCAGGC GAGTGTCTGG TCACAGGACA
1381 GTCCCACTTC AAGAGCTTCG ACAACAGGTA CTTCACCTTC AGTGGGGTCT GCCACTACCT
1441 GCTGGCCCAG GACTGCCAGG ACCACACATT CTCTGTTGTC ATAGAGACTG TCCAGTGTGC
1501 CGATGACCTG GATGCTGTCT GCACCCGCTC GGTCACCGTC CGCCTGCCTG GACATCACAA
1561 CAGCCTTGTG AAGCTGAAGA ATGGGGGAGG AGTCTCCATG GATGGCCAGG ATATCCAGAT
1621 TCCTCTCCTG CAAGGTGACC TCCGCATCCA GCACACCGTG ATGGCCTCCG TGCGCCTCAG
1681 CTACGGGGAG GACCTGCAGA TGGATTCGGA CGTCCGGGGC AGGCTACTGG TGACGCTGTA
1741 CCCCGCCTAC GCGGGGAAGA CGTGCGGCCG TGGCGGGAAC TACAACGGCA ACCGGGGGGA
1801 CGACTTCGTG ACGCCCGCAG GCCTGGCGGA GCCCCTGGTG GAGGACTTCG GGAACGCCTG
1861 GAAGCTGCTC GGGGCCTGCG AGAACCTGCA GAAGCAGCAC CGCGATCCCT GCAGCCTCAA
1921 CCCGCGCCAG GCCAGGTTTG CGGAGGAGGC GTGCGCGCTG CTGACGTCCT CGAAGTTCGA
1981 GCCCTGCCAC CGAGCGGTGG GTCCTCAGCC CTACGTGCAG AACTGCCTCT ACGACGTCTG
2041 CTCCTGCTCC GACGGCAGAG ACTGTCTTTG CAGCGCCGTG GCCAACTACG CCGCAGCCGT
2101 GGCCCGGAGG GGCGTGCACA TCGCGTGGCG GGAGCCGGGC TTCTGTGCGC TGAGCTGCCC
2161 CCAGGGCCAG GTGTACCTGC AGTGTGGGAC CCCCTGCAAC ATGACCTGTC TCTCCCTCTC
2221 TTACCCGGAG GAGGACTGCA ATGAGGTCTG CTTGGAAAGC TGCTTCTCCC CCCCAGGGCT
2281 GTACCTGGAT GAGAGGGGAG ATTGTGTGCC CAAGGCTCAG TGTCCCTGTT ACTATGATGG
2341 TGAGATCTTT CAGCCCGAAG ACATCTTCTC AGACCATCAC ACCATGTGCT ACTGTGAGGA
2401 TGGCTTCATG CACTGTACCA CAAGTGGAGG CCTGGGAAGC CTGCTGCCCA ACCCGGTGCT
2461 CAGCAGCCCC CGGTGTCACC GCAGCAAAAG GAGCCTGTCC TGTCGGCCCC CCATGGTCAA
2521 GTTGGTGTGT CCCGCTGATA ACCCGAGGGC TGAAGGACTG GAGTGTGCCA AAACCTGCCA
2581 GAACTATGAC CTGCAGTGCA TGAGCACAGG CTGTGTCTCC GGCTGCCTCT GCCCGCAGGG
2641 CATGGTCCGG CATGAAAACA GGTGTGTGGC GCTGGAAAGA TGTCCCTGCT TCCACCAAGG
2701 CCAAGAGTAC GCCCCAGGAG AAACCGTGAA AATTGACTGC AACACTTGTG TCTGTCGGGA
2761 CCGGAAGTGG ACCTGCACAG ACCATGTGTG TGATGCCACT TGCTCTGCCA TCGGCATGGC
2821 GCACTACCTC ACCTTCGACG GACTCAAGTA CCTGTTCCCT GGGGAGTGCC AGTATGTTCT
2881 GGTGCAGGAT TACTGCGGCA GTAACCCTGG GACCTTACGG ATCCTGGTGG GGAACGAGGG
2941 GTGCAGCTAC CCCTCAGTGA AATGCAAGAA GCGGGTCACC ATCCTGGTGG AAGGAGGAGA
3001 GATTGAACTG TTTGATGGGG AGGTGAATGT GAAGAAACCC ATGAAGGATG AGACTCACTT
3061 TGAGGTGGTA GAGTCTGGTC AGTACGTCAT TCTGCTGCTG GGCAAGGCAC TCTCTGTGGT
3121 CTGGGACCAC CGCCTGAGCA TCTCTGTGAC CCTGAAGCGG ACATACCAGG AGCAGGTGTG
```

FIGURE 1B

3181 TGGCCTGTGT GGGAATTTTG ATGGCATCCA GAACAATGAT TTCACCAGCA GCAGCCTCCA
3241 AATAGAAGAA GACCCTGTGG ACTTTGGGAA TTCCTGGAAA GTGAACCCGC AGTGTGCCGA
3301 CACCAAGAAA GTACCACTGG ACTCATCCCC TGCCGTCTGC CACAACAACA TCATGAAGCA
3361 GACGATGGTG GATTCCTCCT GCAGGATCCT CACCAGTGAT ATTTTCCAGG ACTGCAACAG
3421 GCTGGTGGAC CCTGAGCCAT TCCTGGACAT TTGCATCTAC GACACTTGCT CCTGTGAGTC
3481 CATTGGGGAC TGCACCTGCT TCTGTGACAC CATTGCTGCT TACGCCCACG TCTGTGCCCA
3541 GCATGGCAAG GTGGTAGCCT GGAGGACAGC CACATTCTGT CCCCAGAATT GCGAGGAGCG
3601 GAATCTCCAC GAGAATGGGT ATGAGTGTGA GTGGCGCTAT AACAGCTGTG CCCCTGCCTG
3661 TCCCATCACG TGCCAGCACC CCGAGCCACT GGCATGCCCT GTACAGTGTG TTGAAGGTTG
3721 CCATGCGCAC TGCCCTCCAG GGAAAATCCT GGATGAGCTT TTGCAGACCT GCATCGACCC
3781 TGAAGACTGT CCTGTGTGTG AGGTGGCTGG TCGTCGCTTG GCCCCAGGAA AGAAAATCAT
3841 CTTGAACCCC AGTGACCCTG AGCACTGCCA AATTTGTAAT TGTGATGGTG TCAACTTCAC
3901 CTGTAAGGCC TGCAGAGAAC CCGGAAGTGT TGTGGTGCCC CCCACAGATG GCCCCATTGG
3961 CTCTACCACC TCGTATGTGG AGGACACGTC GGAGCCGCCC CTCCATGACT TCCACTGCAG
4021 CAGGCTTCTG GACCTGGTTT TCCTGCTGGA TGGCTCCTCC AAGCTGTCTG AGGACGAGTT
4081 TGAAGTGCTG AAGGTCTTTG TGGTGGGTAT GATGGAGCAT CTGCACATCT CCCAGAAGCG
4141 GATCCGCGTG GCTGTGGTGG AGTACCACGA CGGCTCCCAC GCCTACATCG AGCTCAAGGA
4201 CCGGAAGCGA CCCTCAGAGC TGCGGCGCAT CACCAGCCAG GTGAAGTACG CGGGCAGCGA
4261 GGTGGCCTCC ACCAGTGAGG TCTTAAAGTA CACGCTGTTC CAGATCTTTG GCAAGATCGA
4321 CCGCCCGGAA GCGTCTCGCA TTGCCCTGCT CCTGATGGCC AGCCAGGAGC CCTCAAGGCT
4381 GGCCCGGAAT TTGGTCCGCT ATGTGCAGGG CCTGAAGAAG AAGAAAGTCA TTGTCATCCC
4441 TGTGGGCATC GGGCCCCACG CCAGCCTTAA GCAGATCCAC CTCATAGAGA AGCAGGCCCC
4501 TGAGAACAAG GCCTTTGTGT TCAGTGGTGT GGATGAGTTG GAGCAGCGAA GGGATGAGAT
4561 TATCAACTAC CTCTGTGACC TTGCCCCCGA AGCACCTGCC CCTACTCAGC ACCCCCCAAT
4621 GGCCCAGGTC ACGGTGGGTT CGGAGCTGTT GGGGGTTTCA TCTCCAGGAC CCAAAAGGAA
4681 CTCCATGGTC CTGGATGTGG TGTTTGTCCT GGAAGGGTCA GACAAAATTG GTGAGGCCAA
4741 CTTTAACAAA AGCAGGGAGT TCATGGAGGA GGTGATTCAG CGGATGGACG TGGGCCAGGA
4801 CAGGATCCAC GTCACAGTGC TGCAGTACTC GTACATGGTG ACCGTGGAGT ACACCTTCAG
4861 CGAGGCGCAG TCCAAGGGCG AGGTCCTACA GCAGGTGCGG GATATCCGAT ACCGGGGTGG
4921 CAACAGGACC AACACTGGAC TGGCCCTGCA ATACCTGTCC GAACACAGCT TCTCGGTCAG
4981 CCAGGGGGAC CGGGAGCAGG TACCTAACCT GGTCTACATG GTCACAGGAA ACCCCGCTTC
5041 TGATGAGATC AAGCGGATGC CTGGAGACAT CCAGGTGGTG CCCATCGGGG TGGGTCCACA
5101 TGCCAATGTG CAGGAGCTGG AGAAGATTGG CTGGCCCAAT GCCCCCATCC TCATCCATGA
5161 CTTTGAGATG CTCCCTCGAG AGGCTCCTGA TCTGGTGCTA CAGAGGTGCT GCTCTGGAGA
5221 GGGGCTGCAG ATCCCCACCC TCTCCCCCAC CCCAGATTGC AGCCAGCCCC TGGATGTGGT
5281 CCTCCTCCTG GATGGCTCTT CCAGCATTCC AGCTTCTTAC TTTGATGAAA TGAAGAGCTT
5341 CACCAAGGCT TTTATTTCAA GAGCTAATAT AGGGCCCCGG CTCACTCAAG TGTCGGTGCT
5401 GCAATATGGA AGCATCACCA CTATCGATGT GCCTTGGAAT GTAGCCTATG AGAAAGTCCA
5461 TTTACTGAGC CTTGTGGACC TCATGCAGCA GGAGGGAGGC CCCAGCGAAA TTGGGGATGC
5521 TTTGAGCTTT GCCGTGCGAT ATGTCACCTC AGAAGTCCAT GGTGCCAGGC CCGGAGCCTC
5581 GAAAGCGGTG GTTATCCTAG TCACAGATGT CTCCGTGGAT TCAGTGGATG CTGCAGCCGA
5641 GGCCGCCAGA TCCAACCGAG TGACAGTGTT CCCCATTGGA ATCGGGGATC GGTACAGTGA
5701 GGCCCAGCTG AGCAGCTTGG CAGGCCCAAA GGCTGGCTCC AATATGGTAA GGCTCCAGCG
5761 AATTGAAGAC CTCCCCACCG TGGCCACCCT GGGAAATTCC TTCTTCCACA AGCTGTGCTC
5821 TGGGTTTGAT AGAGTTTGCG TGGATGAGGA.TGGGAATGAG AAGAGGCCCG GGGATGTCTG
5881 GACCTTGCCA GACCAGTGCC ACACAGTGAC TTGCCTGCCA GATGGCCAGA CCTTGCTGAA
5941 GAGTCATCGG GTCAACTGTG ACCGGGGGCC AAGGCCTTCG TGCCCCAATG GCCAGCCCCC
6001 TCTCAGGGTA GAGGAGACCT GTGGCTGCCG CTGGACCTGT CCCTGTGTGT GCATGGGCAG
6061 CTCTACCCGG CACATCGTGA CCTTTGATGG GCAGAATTTC AAGCTGACTG GCAGCTGTTC
6121 GTATGTCCTA TTTCAAAACA AGGAGCAGGA CCTGGAGGTG ATTCTCCAGA ATGGTGCCTG
6181 CAGCCCTGGG GCGAAGGAGA CCTGCATGAA ATCCATTGAG GTGAAGCATG ACGGCCTCTC
6241 AGTTGAGCTC CACAGTGACA TGCAGATGAC AGTGAATGGG AGACTAGTCT CCATCCCATA
6301 TGTGGGTGGA GACATGGAAG TCAATGTTTA TGGGACCATC ATGTATGAGG TCAGATTCAA
6361 CCATCTTGGC CACATCTTCA CATTCACCCC CCAAAACAAT GAGTTCCAGC TGCAGCTCAG

FIGURE 1C

```
6421 CCCCAGGACC TTTGCTTCGA AGACATATGG TCTCTGTGGG ATCTGTGATG AGAACGGAGC
6481 CAATGACTTC ATTCTGAGGG ATGGGACAGT CACCACAGAC TGGAAGGCAC TCATCCAGGA
6541 ATGGACCGTA CAGCAGCTTG GGAAGACATC CCAGCCTGTC CATGAGGAGC AGTGTCCTGT
6601 CTCCGAATTC TTCCACTGCC AGGTCCTCCT CTCAGAATTG TTTGCCGAGT GCCACAAGGT
6661 CCTCGCTCCA·GCCACCTTTT ATGCCATGTG CCAGCCCGAC AGTTGCCACC CGAAGAAAGT
6721 GTGTGAGGCG ATTGCCTTGT ATGCCCACCT CTGTCGGACC AAAGGGGTCT GTGTGGACTG
6781 GAGGAGGGCC AATTTCTGTG CTATGTCATG TCCACCATCC CTGGTGTACA ACCACTGTGA
6841 GCATGGCTGC CCTCGGCTCT GTGAAGGCAA TACAAGCTCC TGTGGGGACC AACCCTCGGA
6901 AGGCTGCTTC TGCCCCCCAA ACCAAGTCAT GCTGGAAGGT AGCTGTGTCC CCGAGGAGGC
6961 CTGTACCCAG TGCATCAGCG AGGATGGAGT CCGGCACCAG TTCCTGGAAA CCTGGGTCCC
7021 AGCCCACCAG CCTTGCCAGA TCTGCACGTG CCTCAGTGGG CGGAAGGTCA ACTGTACGTT
7081 GCAGCCCTGC CCCACAGCCA AAGCTCCCAC CTGTGGCCCG TGTGAAGTGG CCCGCCTCCG
7141 CCAGAACGCA GTGCAGTGCT GCCCGGAGTA CGAGTGTGTG TGTGACCTGG TGAGCTGTGA
7201 CCTGCCCCCG GTGCCTCCCT GCGAAGATGG CCTCCAGATG ACCCTGACCA ATCCTGGCGA
7261 GTGCAGACCC AACTTCACCT GTGCCTGCAG GAAGGATGAA TGCAGACGGG AGTCCCCGCC
7321 CTCTTGTCCC CCGCACCGGA CGCCGGCCCT TCGGAAGACT CAGTGCTGTG ATGAGTATGA
7381 GTGTGCATGC AACTGTGTCA ACTCCACGGT GAGCTGCCCG CTTGGGTACC TGGCCTCGGC
7441 TGTCACCAAC GACTGTGGCT GCACCACAAC AACCTGCTTC CCTGACAAGG TGTGTGTCCA
7501 CCGAGGCACC ATCTACCCTG TGGGCCAGTT CTGGGAGGAG GCCTGTGACG TGTGCACCTG
7561 CACGGACTTG GAGGACTCTG TGATGGGCCT GCGTGTGGCC CAGTGCTCCC AGAAGCCCTG
7621 TGAGGACAAC TGCCTGTCAG GCTTCACTTA TGTCCTTCAT GAAGGCGAGT GCTGTGGAAG
7681 GTGTCTGCCA TCTGCCTGTG AGGTGGTCAC TGGTTCACCA CGGGGCGACG CCCAGTCTCA
7741 CTGGAAGAAT GTTGGCTCTC ACTGGGCCTC CCCTGACAAC CCCTGCCTCA TCAATGAGTG
7801 TGTCCGAGTG AAGGAAGAGG TCTTTGTGCA ACAGAGGAAT GTCTCCTGCC CCCAGCTGAA
7861 TGTCCCCACC TGCCCCACGG GCTTCCAGCT GAGCTGTAAG ACCTCAGAGT GTTGTCCCAC
7921 CTGTCACTGC GAGCCCCTGG AGGCCTGCTT GCTCAATGGT ACCATCATTG GGCCGGGGAA
7981 AAGTCTGATG ATTGATGTGT GTACAACCTG CCGCTGCACC GTGCCGGTGG GAGTCATCTC
8041 TGGATTCAAG CTGGAGGGCA GGAAGACCAC CTGTGAGGCA TGCCCCCTGG GTTATAAGGA
8101 AGAGAAGAAC CAAGGTGAAT GCTGTGGGAG ATGTCTGCCT ATAGCTTGCA CCATTCAGCT
8161 AAGAGGAGGA CAGATCATGA CACTGAAGCG TGATGAGACT ATCCAGGATG GCTGTGACAG
8221 TCACTTCTGC AAGGTCAATG AAAGAGGAGA GTACATCTGG GAGAAGAGAG TCACGGGTTG
8281 CCCACCTTTC GATGAACACA AGTGTCTGGC TGAGGGAGGA AAAATCATGA AAATTCCAGG
8341 CACCTGCTGT GACACATGTG AGGAGCCAGA ATGCAAGGAT ATCATTGCCA AGCTGCAGCG
8401 TGTCAAAGTG GGAGACTGTA AGTCTGAAGA GGAAGTGGAC ATTCATTACT GTGAGGGTAA
8461 ATGTGCCAGC AAAGCCGTGT ACTCCATCCA CATGGAGGAT GTGCAGGACC AGTGCTCCTG
8521 CTGCTCGCCC ACCCAGACGG AGCCCATGCA GGTGGCCCTG CGCTGCACCA ATGGCTCCCT
8581 CATCTACCAT GAGATCCTCA ATGCCATCGA ATGCAGGTGT TCCCCCAGGA AGTGCAGCAA
8641 GTGAGGCCAC TGCCTGGATG CTACTGTCGC CTGCCTTACC CGACCTCACT GGACTGGCCA
8701 GAGTGCTGCT CAGTCCTCCT CAGTCCTCCT CCTGCTCTGC TCTTGTGCTT CCTGATCCCA
8761 CAATAAAGGT CAATCTTTCA CCTTGAAAAA AAAAAAAAAA AA
```

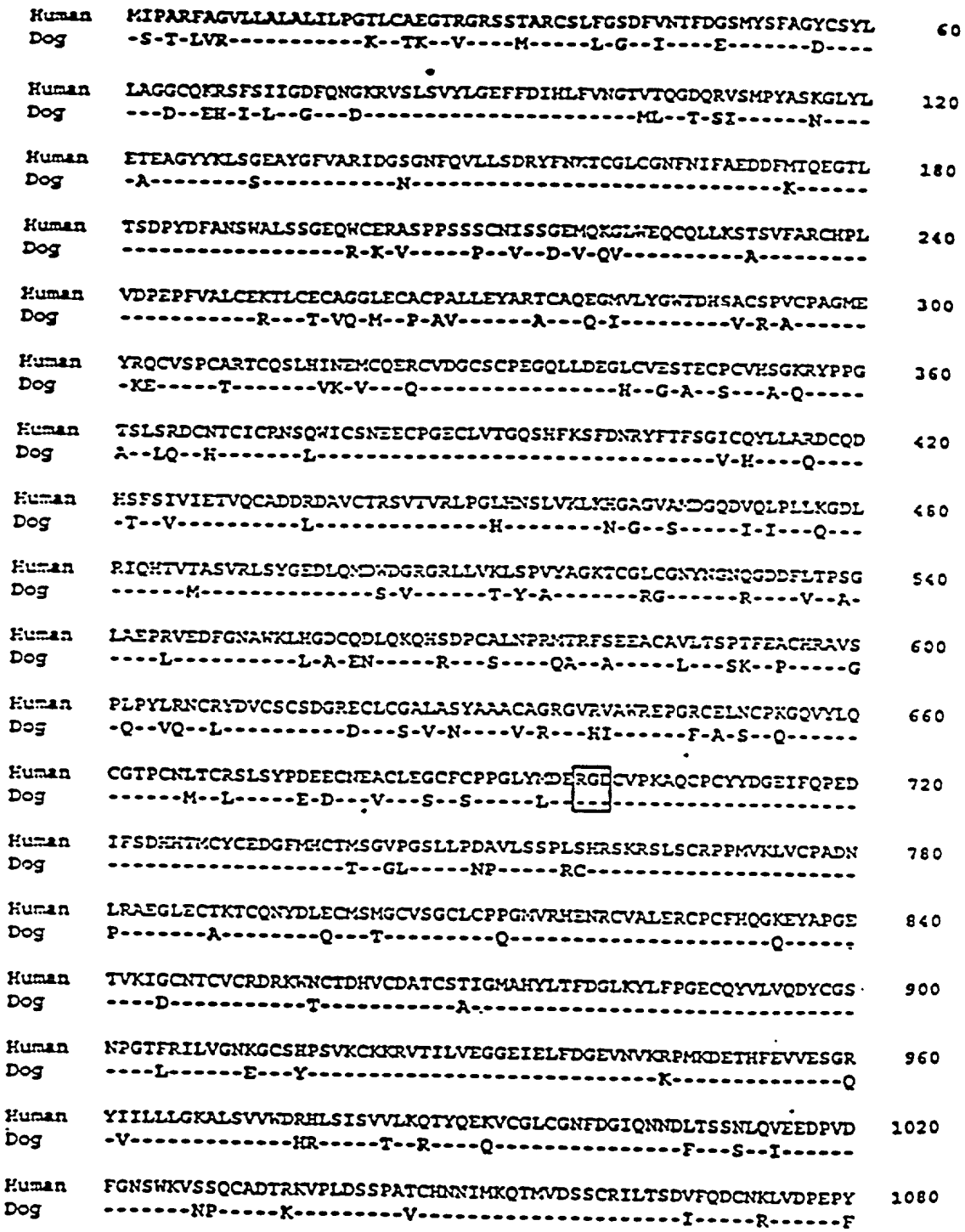

Human MIPARFAGVLLALALILPGTLCAEGTRGRSSTARCSLFGSDFVNTFDGSMYSFAGYCSYL 60
Dog -S-T-LVR----------K--TK--V----M-----L-G--I----E-------D----
Human LAGGCQKRSFSIIGDFQNGKRVSLSVYLGEFFDIHLFVNGTVTQGDQRVSMPYASKGLYL 120
Dog ---D--EH-I-L--G---D----------ML--T-SI------N----
Human ETEAGYYKLSGEAYGFVARIDGSGNFQVLLSDRYFNKTCGLCGNFNIFAEDDFMTQEGTL 180
Dog -A--------S----------N----------K------
Human TSDPYDFANSWALSSGEQWCERASPPSSSCNISSGEMQKGLWEQCQLLKSTSVFARCHPL 240
Dog ----------R-K-V-----P--V--D-V-QV----------A---------
Human VDPEPFVALCEKTLCECAGGLECACPALLEYARTCAQEGMVLYGWTDHSACSPVCPAGME 300
Dog ----------R---T-VQ-M--P-AV------A---Q-I---------V-R-A------
Human YRQCVSPCARTCQSLHINEMCQERCVDGCSCPEGQLLDEGLCVESTECPCVHSGKRYPPG 360
Dog -KE-----T-------VK-V---Q----------H--G-A--S---A-Q-----
Human TSLSRDCNTCICRNSQWICSNEECPGECLVTGQSHFKSFDNRYFTFSGICQYLLARDCQD 420
Dog A--LQ--H-------L----------V-H----Q----
Human HSFSIVIETVQCADDRDAVCTRSVTVRLPGLHNSLVKLKHGAGVAMDGQDVQLPLLKGDL 480
Dog -T--V----------L----------H--------N-G--S-----I-I---Q---
Human RIQHTVTASVRLSYGEDLQMQWDGRGRLLVKLSPVYAGKTCGLCGNYNGNQGDDFLTPSG 540
Dog ------M----------S-V------T-Y-A-------RG------R----V--A-
Human LAEPRVEDFGNAWKLHGDCQDLQKQHSDPCALNPRMTRFSEEACAVLTSPTFEACHRAVS 600
Dog ----L----------L-A-EN-----R---S----QA--A-----L---SK--P-----G
Human PLPYLRNCRYDVCSCSDGRECLCGALASYAAACAGRGVRVAWREPGRCELNCPKGQVYLQ 660
Dog -Q--VQ--L----------D---S-V-N----V-R---HI------F-A-S--Q------
Human CGTPCNLTCRSLSYPDEECNEACLEGCFCPPGLYMDERGDCVPKAQCPCYYDGEIFQPED 720
Dog ------M--L-----E-D---V---S--S-----L-[---]----------
Human IFSDHHTMCYCEDGFMHCTMSGVPGSLLPDAVLSSPLSHRSKRSLSCRPPMVKLVCPADN 780
Dog ----------T--GL-----NP-----RC----------
Human LRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCVALERCPCFHQGKEYAPGE 840
Dog P-------A--------Q---T---------Q----------Q-----
Human TVKIGCNTCVCRDRKWNCTDHVCDATCSTIGMAHYLTFDGLKYLFPGECQYVLVQDYCGS 900
Dog ----D----------T----------A----------
Human NPGTFRILVGNKGCSHPSVKCKKRVTILVEGGEIELFDGEVNVKRPMKDETHFEVVESGR 960
Dog ----L------E---Y----------K----------Q
Human YIILLLGKALSVVWDRHLSISVVLKQTYQEKVCGLCGNFDGIQNNDLTSSNLQVEEDPVD 1020
Dog -V----------HR-----T--R----Q----------F---S--I------
Human FGNSWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRILTSDVFQDCNKLVDPEPY 1080
Dog -------NP-----K---------V----------I-----R------F

FIGURE 2A

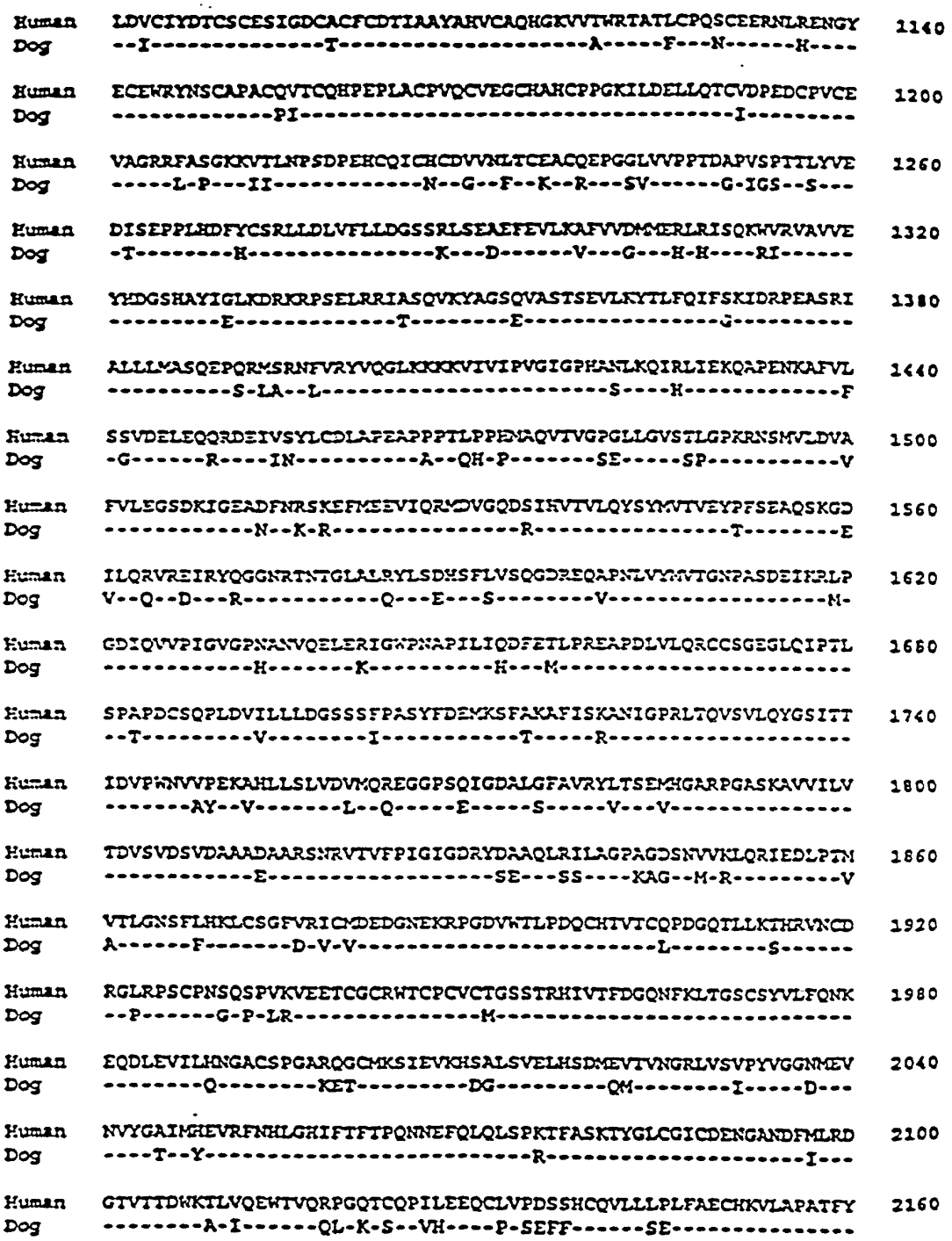

Human LDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKVVTWRTATLCPQSCEERNLRENGY 1140
Dog --I----------T----------A-----F---N------H----
Human ECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKILDELLQTCVDPEDCPVCE 1200
Dog ----------PI----------I---------
Human VAGRRFASGKKVTLNPSDPEHCQICHCDVVNLTCEACQEPGGLVVPPTDAPVSPTTLYVE 1260
Dog -----L-P---II----------N--G--F--K--R---SV------G-IGS--S---
Human DISEPPLHDFYCSRLLDLVFLLDGSSRLSEAEFEVLKAFVVDMMERLRISQKWVRVAVVE 1320
Dog -T--------H----------K---D------V---G---H-H----RI------
Human YHDGSHAYIGLKDRKRPSELRRIASQVKYAGSQVASTSEVLKYTLFQIFSKIDRPEASRI 1380
Dog ---------E----------T--------E----------G----------
Human ALLLMASQEPQRMSRNFVRYVQGLKKKKVIVIPVGIGPHANLKQIRLIEKQAPENKAFVL 1440
Dog ----------S-LA--L----------S----H----------F
Human SSVDELEQQRDEIVSYLCDLAPEAPPPTLPPHMAQVTVGPGLLGVSTLGPKRNSMVLDVA 1500
Dog -G------R----IN----------A--QH-P-------SE-----SP----------V
Human FVLEGSDKIGEADFNRSKEFMEEVIQRMDVGQDSIHVTVLQYSYMVTVEYPFSEAQSKGD 1560
Dog ----------N--K-R----------R----------T--------E
Human ILQRVREIRYQGGNRTNTGLALRYLSDHSFLVSQGDREQAPNLVYMVTGNPASDEIKRLP 1620
Dog V--Q--D---R----------Q---E---S--------V----------M-
Human GDIQVVPIGVGPNANVQELERIGWPNAPILIQDFETLPREAPDLVLQRCCSGEGLQIPTL 1680
Dog ----------H-------K----------H---M----------
Human SPAPDCSQPLDVILLLDGSSSFPASYFDEMKSFAKAFISKANIGPRLTQVSVLQYGSITT 1740
Dog --T---------V--------I----------T-----R----------
Human IDVPWNVVPEKAHLLSLVDVMQREGGPSQIGDALGFAVRYLTSEMHGARPGASKAVVILV 1800
Dog -------AY--V-------L--Q-----E-----S-----V---V----------
Human TDVSVDSVDAAADAARSNRVTVFPIGIGDRYDAAQLRILAGPAGDSNVVKLQRIEDLPTM 1860
Dog ----------E----------SE---SS----KAG--M-R---------V
Human VTLGNSFLHKLCSGFVRICMDEDGNEKRPGDVWTLPDQCHTVTCQPDGQTLLKTHRVNCD 1920
Dog A------F-------D-V-V----------L--------S------
Human RGLRPSCPNSQSPVKVEETCGCRWTCPCVCTGSSTRHIVTFDGQNFKLTGSCSYVLFQNK 1980
Dog --P------G-P-LR----------M----------
Human EQDLEVILHNGACSPGARQGCMKSIEVKHSALSVELHSDMEVTVNGRLVSVPYVGGNMEV 2040
Dog --------Q--------KET---------DG---------QM--------I-----D---
Human NVYGAIMHEVRFNHLGHIFTFTPQNNEFQLQLSPKTFASKTYGLCGICDENGANDFMLRD 2100
Dog ----T--Y----------R----------I---
Human GTVTTDWKTLVQEWTVQRPGQTCQPILEEQCLVPDSSHCQVLLLPLFAECHKVLAPATFY 2160
Dog --------A-I------QL-K-S--VH----P-SEFF------SE----------

FIGURE 2B

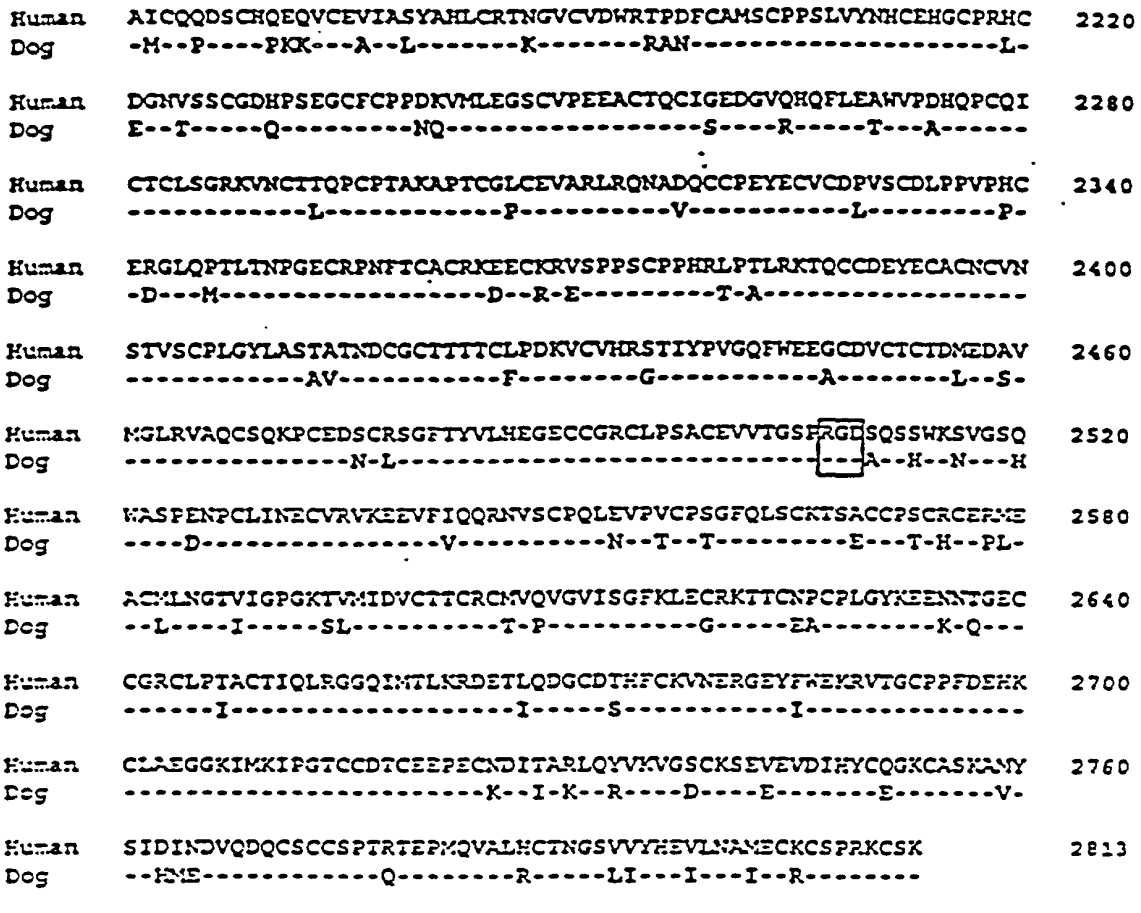

Human AICQQDSCHQEQVCEVIASYAHLCRTNGVCVDWRTPDFCAMSCPPSLVYNHCEHGCPRHC 2220
Dog -M--P----PKK---A--L-------K-------RAN----------L-
Human DGNVSSCGDHPSEGCFCPPDKVMLEGSCVPEEACTQCIGEDGVQHQFLEAWVPDHQPCQI 2280
Dog E--T-----Q---------NQ----------S----R-----T---A------
Human CTCLSGRKVNCTTQPCPTAKAPTCGLCEVARLRQNADQCCPEYECVCDPVSCDLPPVPHC 2340
Dog ----------L----------P----------V----------L---------P-
Human ERGLQPTLTNPGECRPNFTCACRKEECKRVSPPSCPPHRLPTLRKTQCCDEYECACNCVN 2400
Dog -D---M----------D--R-E---------T-A----------
Human STVSCPLGYLASTATNDCGCTTTTCLPDKVCVHRSTIYPVGQFWEEGCDVCTCTDMEDAV 2460
Dog ----------AV----------F--------G----------A--------L--S-
Human MGLRVAQCSQKPCEDSCRSGFTYVLHEGECCGRCLPSACEVVTGSPRGDSQSSWKSVGSQ 2520
Dog ----------N-L----------A--H--N---H
Human WASPENPCLINECVRVKEEVFIQQRNVSCPQLEVPVCPSGFQLSCKTSACCPSCRCERME 2580
Dog ----D----------V----------N--T--T---------E---T-H--PL-
Human ACMLNGTVIGPGKTVMIDVCTTCRCMVQVGVISGFKLECRKTTCNPCPLGYKEENNTGEC 2640
Dog --L----I-----SL----------T-P----------G-----EA--------K-Q---
Human CGRCLPTACTIQLRGGQIMTLKRDETLQDGCDTHFCKVNERGEYFWEKRVTGCPPFDEHK 2700
Dog ------I----------I-----S----------I----------
Human CLAEGGKIMKIPGTCCDTCEEPECNDITARLQYVKVGSCKSEVEVDIHYCQGKCASKAMY 2760
Dog ----------K--I-K--R----D----E-------E-------V-
Human SIDINDVQDQCSCCSPTRTEPMQVALHCTNGSVVYHEVLNAMECKCSPRKCSK 2813
Dog --HME----------Q--------R-----LI---I---I--R--------

FIGURE 2C

FIG. 2

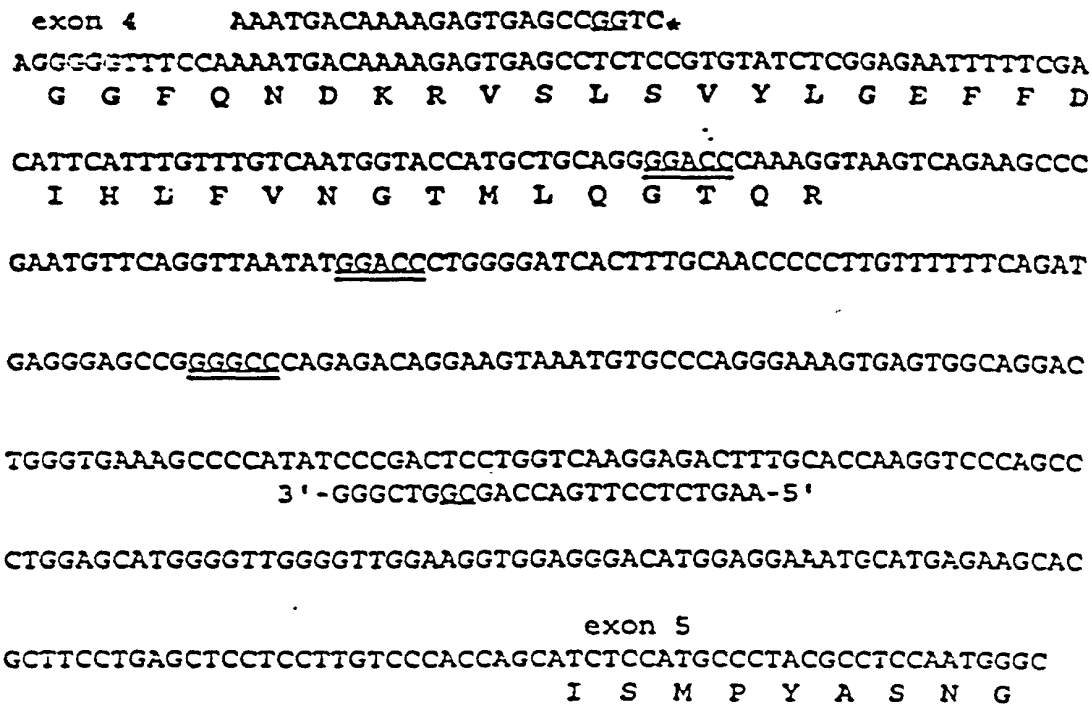


FIGURE 4

Fig. 5.

Normal Allele

Exon 43 Intron 43 Exon 44

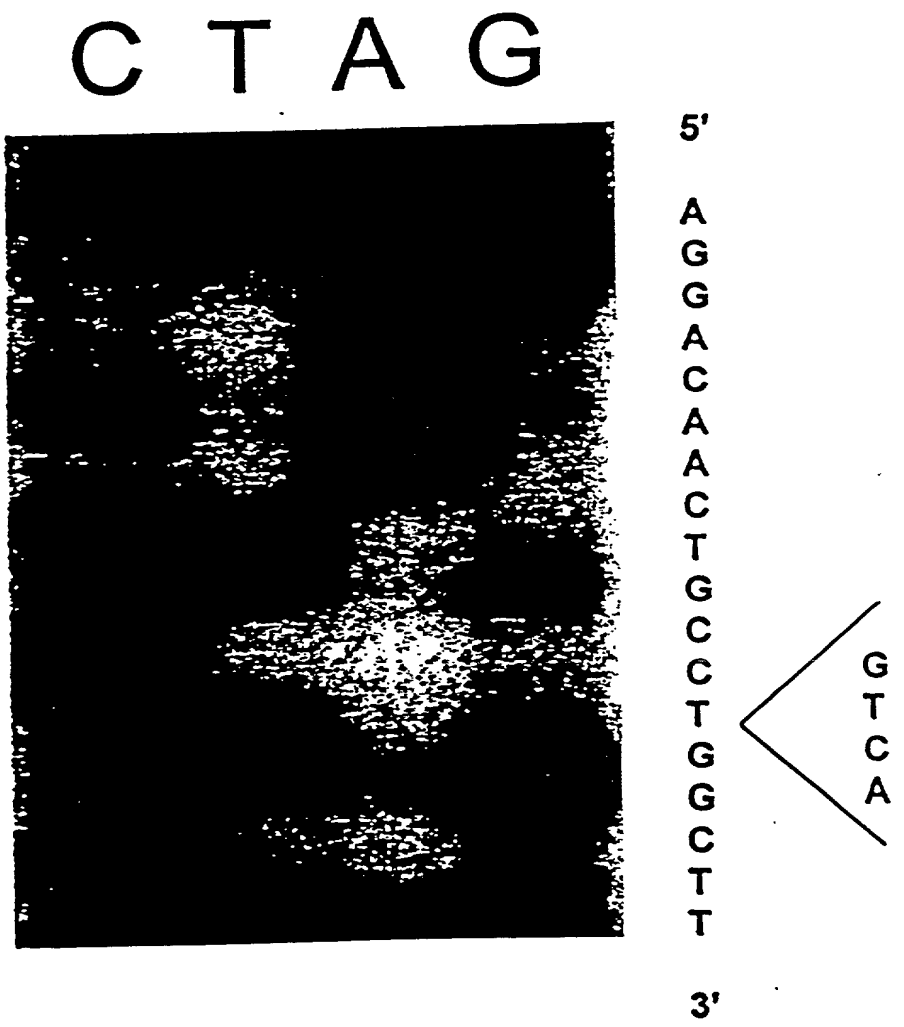
```
                          *
AGGACAACTGCCTGCCTGTCGgtgagtgggg ... GGCTTCACTTAT
                    |||||||
                   AGGTRAGT  Donor Consensus
```

Mutant Allele

```
                          *
AGGACAACTGCCTGCCTgtcagtgagtgggg ... GGCTTCACTTAT
                   ||  |||
                  AGGTRAGT  Donor Consensus
```

Figure 6

Figure 7



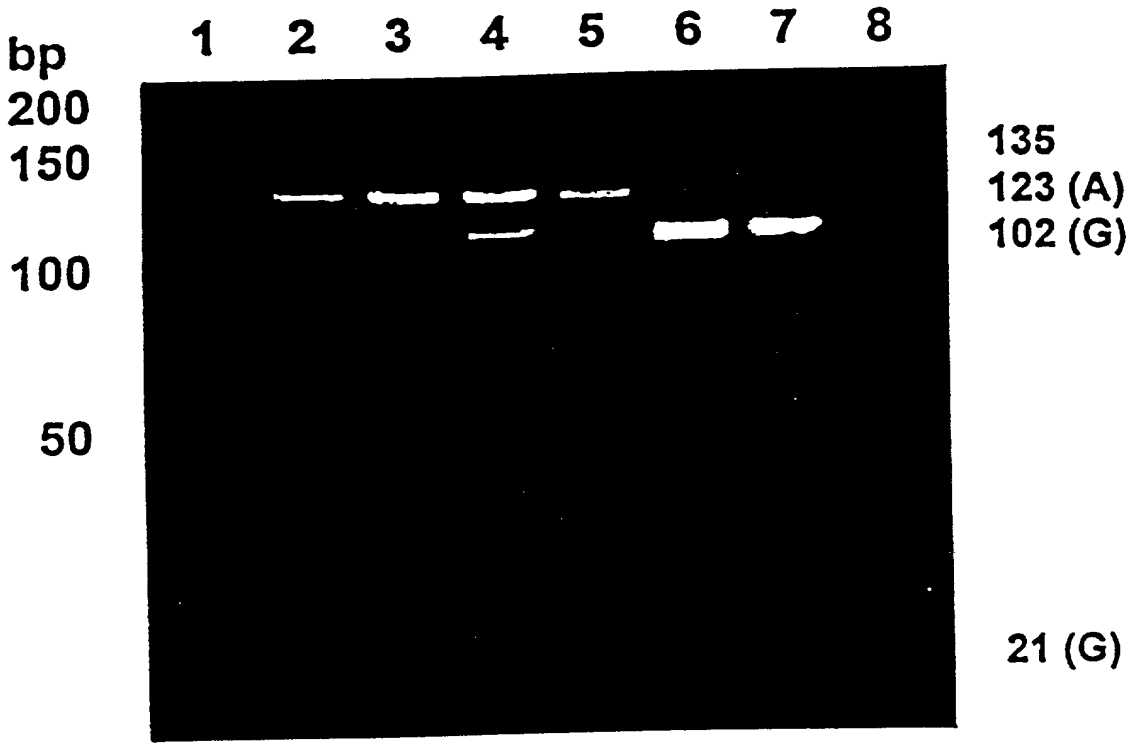
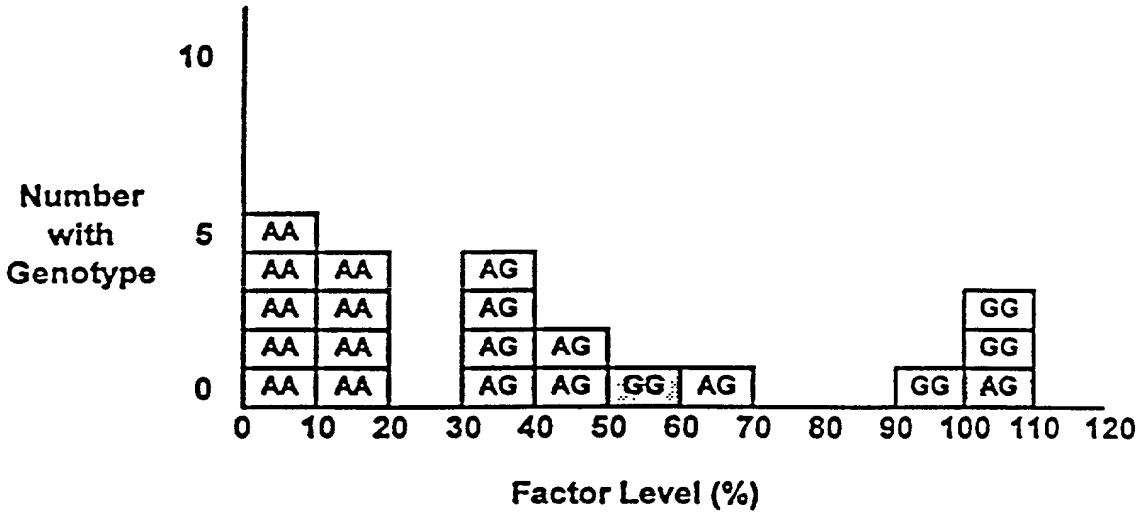


Figure 8

Figure 9


Number with Genotype
10
5
0
AA
AG
GG
0 10 20 30 40 50 60 70 80 90 100 110 120
Factor Level (%)

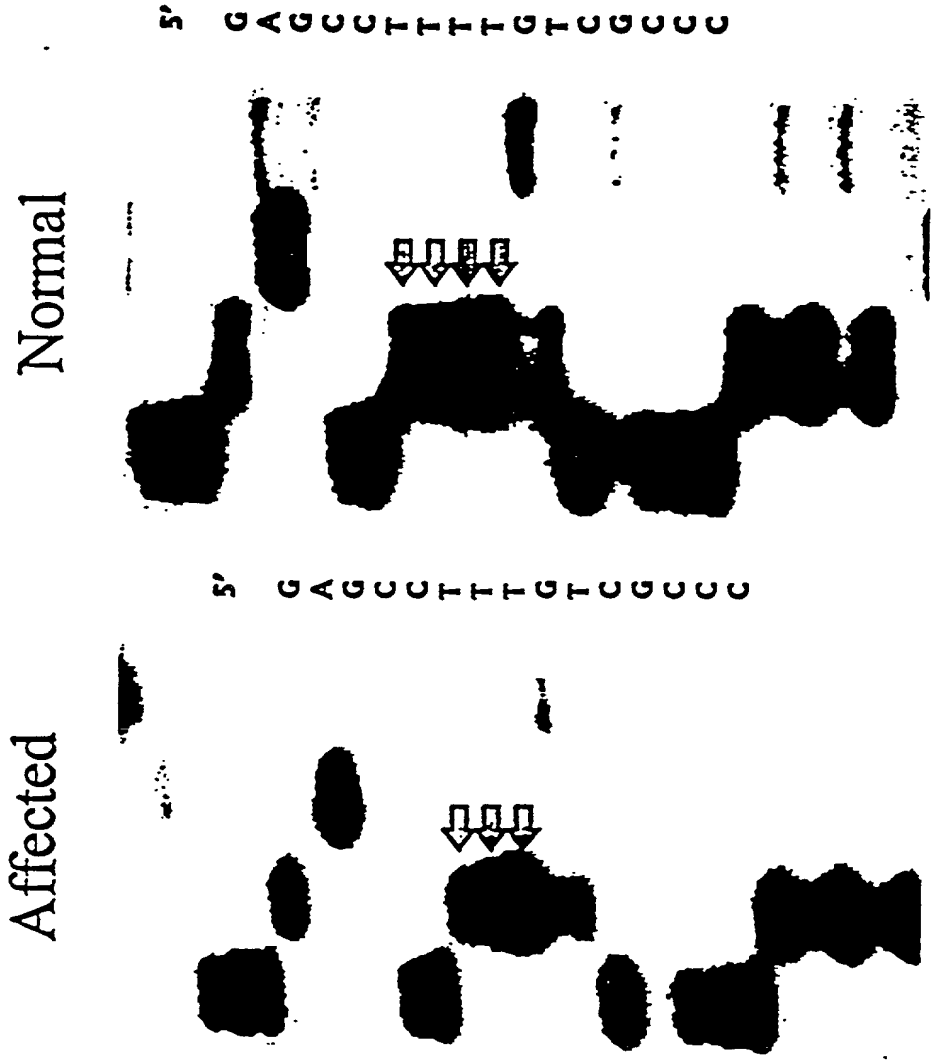


Figure 10

Exon 7

```
V  L  W  E  Q  C  Q  L  L  K  S  A  S  V  F  A  R  C  H  P  L  V
GTCCTGTGGGAGCAGTGCCAGCTCCTGAAGAGTGCCTCGGTGTTTGCCCGCTGCCACCCGCTGGTG
 TCCTGTGGGAGCAGTGCCAG
DVWFEX7D   GCNNNNNNNGC Mwo I

D  P  E  P  F  V  A  L  C  E  R  T  L  C  T  C  V  Q  G  M  E  C
GACCCTGAGCCTTTTGTCGCCCTGTGTGAAAGGACTCTGTGCACCTGTGTCCAGGGGATGGAGTGC
          GCNNNN-NNNGC  Mwo I
                Δ735

P  C  A  V  L  L  E  Y  A  R  A  C  A  Q  Q  G  I  V  L  Y  G  W
CCTTGTGCGGTCCTCCTGGAGTACGCCCGGGCCTGTGCCCAGCAGGGAATTGTGCTGTACGGCTGG
                                                         ATGCCGACC

T  D  H  S  V  C  R
ACCGACCACAGCGTCTGCCG
TGGCTGGTG-5'
DVWFEX7U
```

Figure 11

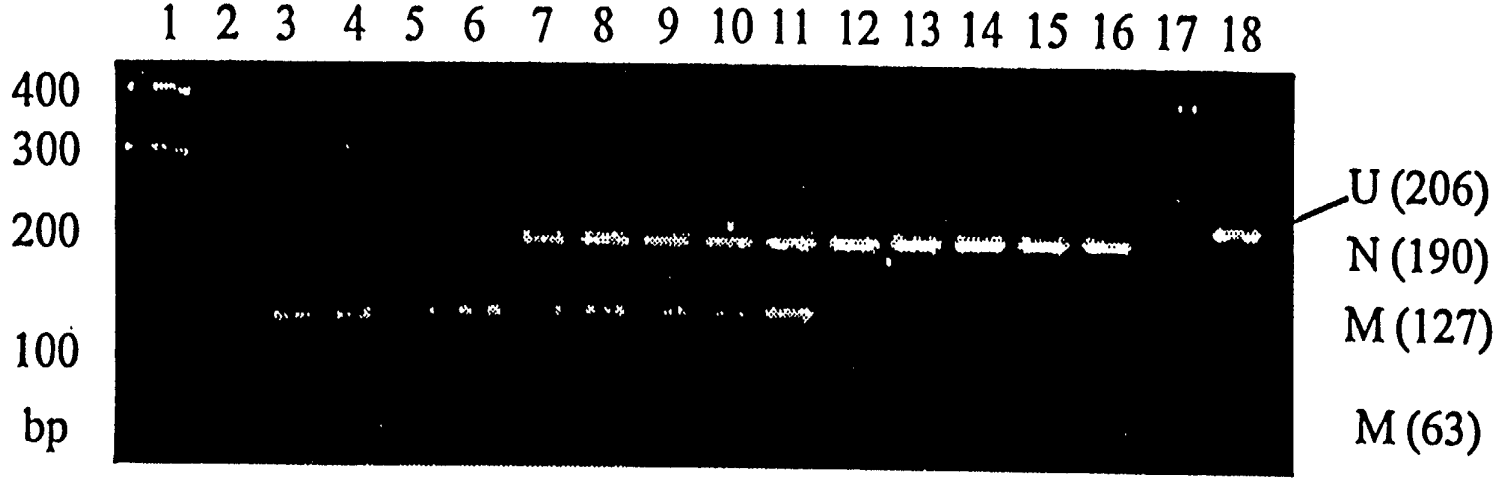

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
400
300
200
100
bp
U (206)
N (190)
M (127)
M (63)

Figure 12

Customer Number: 000959

Attorney's
Docket
Number UMV-1226CPPCUS

Declaration, Petition and Power of Attorney for Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

DNA ENCODING CANINE VON WILLEBRAND FACTOR AND METHODS OF USE
the specification of which

(check one)

X is attached hereto.

_ was filed on _____ as

Application Serial No. _____

and was amended on _____.
(if applicable)

I do not know and do not believe that the subject matter of this application was known or used by others in the United States or patented or described in a printed publication in any country before my invention thereof, or patented or described in a printed publication in any country or in public use or on sale in the United States more than one year prior to the date of this application, or first patented or caused to be patented or made the subject of an inventor's certificate by me or my legal representatives or assigns in a country foreign to the United States prior to the date of this application on an application filed more than twelve months (six months if this application is for a design) before the filing of this application; and I acknowledge my duty to disclose information of which I am aware which is material to the examination of this application, that no application for patent or inventor's certificate on the subject matter of this application has been filed by me or my representatives or assigns in any country foreign to the United States, except those identified below, and that I have reviewed and understand the contents of the specification, including the claims as amended by any amendment referred to herein.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

CLAIM OF BENEFIT OF EARLIER FOREIGN APPLICATION(S)

I hereby claim priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below, and have also identified below any foreign application(s) for patent or inventor's certificate filed by me on the same subject matter having a filing date before that of the application(s) from which priority is claimed.

Check one:

___ no such applications have been filed.

X_ such applications have been filed as follows

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Country	Application Number	Date of Filing (month,day,year)	Priority Claimed Under 35 USC 119
PCT	PCT/US99/18153	August 10, 1999	X Yes No _
			_ Yes No _
			_ Yes No _
			_ Yes No _
			_ Yes No _

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

CLAIM FOR BENEFIT OF U.S. PROVISIONAL APPLICATION(S)

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

______________________	______________________
(Application Serial No.)	(Filing Date)
______________________	______________________
(Application Serial No.)	(Filing Date)

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of any earlier United States application(s) or PCT international application(s) designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the earlier application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date(s) of the earlier application(s) and the national or PCT international filing date of this application. As to subject matter of this application which is common to my earlier application(s), if any, described below, I do not know and do not believe that the same was known or used by others in the United States or patented or described in a printed publication in any country before my invention thereof, or patented or described in a printed publication in any country or in public use or on sale in the United States more than one year prior to the date(s) of said earlier application(s), or first patented or caused to be patented or made the subject of an inventor's certificate by me or my legal representatives or assigns in a country foreign to the United States prior to the date(s) of said earlier application(s) on an application filed more than twelve months (six months if this application is for a design) before the filing of said earlier application(s); and I acknowledge that no application for patent or inventor's certificate on said subject matter has been filed by me or my representatives or assigns in any country foreign to the United States except those identified herein.

(Application Serial No.)	(Filing Date)	(Status) (patented,pending,aband.)
09/132,652	August 11, 1998	Patented

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

James E. Cockfield	Reg. No. 19,162	Nicholas P. Triano III	Reg. No. 36,397
Thomas V. Smurzynski	Reg. No. 24,798	Peter C. Lauro	Reg. No. 32,360
Ralph A. Loren	Reg. No. 29,325	DeAnn F. Smith	Reg. No. 36,683
Giulio A. DeConti, Jr.	Reg. No. 31,503	William D. DeVaul	Reg. No. 42,483
Ann Lamport Hammitte	Reg. No. 34,858	David J. Rikkers	Reg. No. 43,882
Elizabeth A. Hanley	Reg. No. 33,505	Chi Suk Kim	Reg. No. 42,728
Amy E. Mandragouras	Reg. No. 36,207	Maria Laccotripe Zacharakis	Limited Recognition Under 37 C.F.R. § 10.9(b)
Anthony A. Laurentano	Reg. No. 38,220		
Jane E. Remillard	Reg. No. 38,872	Debra J. Milasincic	Reg. No. P46,931
Jeremiah Lynch	Reg. No. 17,425	David R. Burns	Reg. No. P46,590
Kevin J. Canning	Reg. No. 35,470	Sean D. Detweiler	Reg. No. 42,482
Jeanne M. DiGiorgio	Reg. No. 41,710	Peter S. Stecher	Reg. No. P47,259
Megan E. Williams	Reg. No. 43,270		

Send Correspondence to DeAnn F. Smith at **Customer Number: 000959** whose address is:

Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109

Direct Telephone Calls to: (name and telephone number)

DeAnn F. Smith , (617) 227-7400

Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements ma on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Patrick J. Venta
Inventor's signature Date
Residence 9646 Rolling Green, Pinckney, Michigan 48169
Citizenship United States of America
Post Office Address (if different)

Full name of second inventor, if any George J. Brewer	
Inventor's signature	Date
Residence	
Citizenship United States of America	
Post Office Address (if different)	

Full name of third inventor, if any Vilma Yuzbasiyan-Gurkan	
Inventor's signature	Date
Residence 3101 Dexter Road, Ann Arbor, Michigan 48103	
Citizenship United States of America	
Post Office Address (if different)	

Full name of fourth inventor, if any William D. Schall	
Inventor's signature	Date
Residence 3150 S. Williamston, Williamston, Michigan 48895	
Citizenship United States of America	
Post Office Address (if different)	